Please add the following new claim:

D4 Sub E2 --72. (NEW) The method of claim 28, wherein the B7-2 is human B7-2.--

ADD E3

REMARKS

The specification and Abstract of the Disclosure have been amended to correct minor informalities. The Abstract of the Disclosure has been replaced to more accurately describe the present invention. Applicants submit herewith a "Version with Markings to Show Changes Made to the Specification and Abstract of the Disclosure" as Appendix A which indicates the specific amendments made to the Application. No new matter has been added by way of these amendments.

Claims 63-64 and 68-69 have been cancelled without prejudice herein. New claim 72 is added. Accordingly, claims 28, 65, 66, 67, and 70-72 are presently pending in the application. It is Applicants' understanding that a search has been completed for the species of the agent which inhibits B7-2 binding with its natural ligand which is an antibody which recognizes B7-2. It is further Applicants understanding that this election is for searching purposes only and upon allowance of the elected claims, the generic claims will also be searched and Applicants will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 C.F.R. §1.141. Applicants submit that Claims 28, 67, 70, 71, and 72 are generic claims.

Filing Date of the Instant Claims

The Examiner has deemed the filing date of the instant claims to be the filing date of the priority application USSN 08/280,757, filed 7/26/94. The Examiner states that the previous priority applications do not provide sufficient written description for the claimed methods of

inhibiting an interaction of a B lymphocyte antigen B7-2 with its natural ligand on the surface of an immune cell, an agent which inhibits B7-2 binding with its natural ligand, antibody reactive with CD28, antibody reactive with CTLA4, antibody reactive with a cytokine, a CTLA4Ig fusion protein, a CD28Ig fusion protein, and an immunosuppressive drug. More specifically, referring to these limitations, the Examiner states:

> While USSN 08/101,624 discloses monoclonal or chimeric antibodies reacted with the described B7 molecules for immunotherapy as well as monomeric B7 molecules for downregulating or preventing B lymphocyte antigen functions; USSN 08/101,624 does not disclose the "limitations" indicated above.

It is Applicants' position that earlier priority documents (e.g., USSN 08/101,624) disclose inhibiting an interaction of a B lymphocyte antigen B7-2 with its natural ligand on the surface of an immune cell, an agent which inhibits B7-2 binding with its natural ligand, antibody reactive with CD28, antibody reactive with CTLA4, antibody reactive with a cytokine, a CTLA4Ig fusion protein, a CD28Ig fusion protein, and an immunosuppressive drug. In response, Applicants provide the following detailed analysis of the support for these limitations in USSN 08/101,624.

> Inhibiting an Interaction of a B Lymphocyte Antigen B7-2 With its Natural Ligand on the Surface of an Immune Cell and An Agent Which Inhibits B7-2 Binding With Its Natural Ligand

Support for the limitation "inhibiting an interaction of a B lymphocyte antigen B7-2 with its natural ligand on the surface of an immune cell" is found throughout USSN 08/101,624. More specifically, direct support is found at least in the Summary on page 5, line 19-28:

> The invention also provides methods for inducing tolerance in a subject by, for example, blocking the functional interaction of the novel B lymphocyte antigens of the invention, e.g., B7-2 and B7-3, to their natural ligand(s) on T cells or other immune system cells, to thereby block co-stimulation through the receptor-ligand pair. In one embodiment, molecules that can be used to block the interaction of the human B7-2 antigen to its natural ligands (e.g., CTLA4 and CD28) include soluble B7-2, antibodies that block the binding of B7-2 to its ligands and fail to deliver a co-stimulatory signal (so called "blocking antibodies") and B7-2-Ig

> fusion proteins, which can be produced in accordance with the teaching of the present invention.

This passage also provides support for the use of agents which inhibit B7-2 binding with its natural ligand. Specific agents supported are antibodies which bind B7-2, antibodies which bind CTLA4 and CD28 (blocking antibodies), and B7-2-Ig fusion proteins.

The concept of inhibiting an interaction of a B lymphocyte antigen B7-2 with its natural ligand on the surface of an immune cell is also discussed on page 11, line 8-13:

> Nucleic acid fragments which encode polypeptides which retain the ability to bind to their natural ligand(s) on T cells and either amplify or ***block*** activated T cell mediated immune responses (as evidenced by, for example, lymphokine production and/or T cell proliferation by T cells that have received a primary activation signal) are considered within the scope of the invention. (emphasis added)

This passage also provides support for the specific agent nucleic acid fragments which encode polypeptides which retain the ability to bind to their natural ligands to block an immune response.

Additional agents which might block the interaction of B7-2 with its natural receptor on the surface of an immune cell are discussed at least on page 22, line 1-4, under the heading of "Modifications of Nucleic Acid and Amino Acid Sequences," which reads:

> Furthermore, the DNA sequence of B lymphocyte antigens can be modified by genetic techniques to produce proteins or peptides with altered amino acid sequences. Such sequences are considered within the scope of the present invention, ***where the expressed protein is capable of*** either enhancing or ***blocking activated T cell mediated immune responses and immune function***. (emphasis added)

Polypeptide fragments, mutants and variants of B lymphocyte antigens are discussed at least in the second paragraph of page 23, as are soluble, monomeric forms of the B7-2 protein. These agents are discussed in terms of their ability to block T cell activation (as set forth throughout the specification, this occurs by blocking the interaction between B7-2 and its natural ligand):

> ***Fragments, mutants and variants of B lymphocyte antigens*** that retain the ability to bind to their natural ligand(s) on T cells and either amplify or ***block*** activated T cell

> mediated immune responses, as evidenced by, for example, lymphokine production and/or T cell proliferation by T cells that have received a primary activation signal are considered within the scope of the invention. More specifically, B7-2 proteins and peptides that bind to T lymphocytes, for example $CD28^+$ cells, may be capable of delivering a costimulatory signal to the T lymphocytes, which, when transmitted in the presence of antigen and class II MHC, or other material capable of transmitting a primary signal to the T cell, results in activation of lymphokine genes within the T cell. Such B7-2 proteins can be considered to retain the essential characteristics of the B7-2 cell surface antigen. Alternatively, ***B7-2 proteins, particularly soluble, monomeric forms of the B7-2 protein, may retain the ability to bind to their natural ligand(s) on $CD28^+$ T cells but, perhaps because of insufficient cross-linking with the ligand, fail to deliver the secondary signal essential for enhanced lymphokine production and cell division. Such proteins, which provide a means to induce a state of anergy or tolerance in the cells, are also considered within the scope of the invention.*** (emphasis added)

Further support for inhibition of interaction of B7-2 with its natural ligand is found at least on page 27, line 6-10:

> For example, ***B7-2 proteins and peptides***, including ***soluble, monomeric*** forms of the B7-2 antigen, that fail to deliver a costimulatory signal to T cells that have received a primary activation signal, can be used to block the B7-2 ligand(s) on T cells and thereby provide a specific means by which to induce tolerance in a subject.

One of skill in the art will immediately recognize that the term "block" as used, refers to blocking of B7-2 binding to its natural ligand on the cell surface, thus providing implicit support for Applicants' claims. The above quoted passage also provides additional support for B7-2 proteins as agents which inhibit binding, in particular for B7-2 proteins which are soluble, and monomeric.

The following quoted passage provides further support for the specific agents of an antibody which binds to B7-2 used to block B7-2, page 34, line 9-11:

> For example, antibodies reactive with the B7-2 antigen can be used to isolate the naturally-occurring or native form of B7-2 or to block B7-2 function.

The Term "Immune Cell"

Support for use of the term "immune cell" in the claims is found at least in the specification on page 5, line 19-23, which states:

> The invention also provides methods for inducing tolerance in a subject by, for example, blocking the functional interaction of the novel B lymphocyte antigens of the invention, e.g., B7-2 and B7-3, to their natural ligand(s) on T cells or ***other immune system cells***, to thereby block co-stimulation through the receptor-ligand pair. (emphasis added)

Combination of Immunomodulating Agents

Support for contacting an immune cell with a combination of immunomodulating agents is found, for example, on page 28, line 6-8, which reads:

> It may also be necessary to block the function of a combination of B lymphocyte antigens to achieve sufficient immunosuppression or tolerance in a subject.

Support for contacting an immune cell with the specific immunomodulating agents of an antibody which binds CD28 and also a CTLA4-Ig fusion protein to an immune cell is found for instance in Example 1, which exemplifies the use of an anti-CD28 antibody and also a CTLA4-Ig fusion protein. This is indicated, for example, by the following passage on page 40, line 18-22:

> Both proliferation and IL-2 secretion were totally inhibited by blocking the B7-1 molecule on CHO cells with either anti-B7-1 monoclonal antibody or by a fusion protein for its high affinity receptor, CTLA4. Similarly, proliferation and IL-2 secretion were abrogated by blocking B7-1 signaling via CD28 with Fab anti-CD28 monoclonal antibody.

Further support for contacting an immune cell with a combination of immunomodulating agents is found, for example, on page 27, line 32, to page 28, line 4:

> The administration of a soluble, monomeric form of B7-2 ***alone or in conjunction with a monomeric form of another B lymphocyte antigen (e.g., B7-1, B7-3) or blocking antibody***, prior to transplantation can lead to the binding of the monomeric antigen(s) to its natural ligand(s) on T cells without transmitting the corresponding costimulatory signal and thus blocks the ligand(s) on T cells. Blocking B lymphocyte antigen function

in this manner prevents T cell lymphokine synthesis and thus acts as an immunosuppressant. (emphasis added)

Title of the Invention

The Examiner has objected to the title of the invention as not descriptive and requires a new title which is clearly indicative of the claimed invention. In response, Applicants have amended the title to now read "Methods for Inhibiting The Interaction of B7-2 With Its Natural Ligand."

Abstract of the Disclosure

The Examiner has objected to the Abstract of the Disclosure as not adequately describing the claimed invention. In response, Applicants have replaced the Abstract of the Disclosure with a new Abstract of the Disclosure which more accurately describes the claimed invention.

Objections to the Drawings

The Examiner has indicated that the drawings submitted fail to comply with the necessary requirements. In response, Applicants agree to supply formal drawings which comply with the requirements of 37 C.F.R. 1.84 upon issuance of a notice of allowability.

Requested Corrections to the Specification

The Examiner has required that the application be reviewed and all spelling, TRADEMARKS, and like errors be corrected. Applicants have made every effort to detect and correct such errors in the application. Applicants submit that the trademarks known to Applicants are capitalized, that the proprietary nature of the marks has been respected, and that every effort has been made to prevent their use in any manner which might adversely affect their value as trademarks.

The Pending Claims

The pending claims are directed to methods for inhibiting an interaction of a B lymphocyte antigen, B7-2, with its natural ligand on the surface of an immune cell, comprising contacting the

immune cell with an agent which inhibits B7-2 binding with its natural ligand to thereby inhibit interaction of B7-2 with its natural ligand on the immune cell. In another embodiment, the agent is an antibody that recognizes B7-2. In one embodiment, the method further comprises contacting the immune cell with an agent that blocks the interaction of B7-1 with its natural ligand. In one embodiment, the step of contacting is performed *in vitro*. In another embodiment, the step of contacting is performed *in vivo*.

Rejection of Claims 28, 66, 67, 70, and 71 Under 35 U.S.C. § 112, First Paragraph as Non-enabled by the Specification

The Examiner has rejected claims 28, 66, 67, 70, and 71 under U.S.C. § 112, first paragraph as failing to adequately teach how to make and/or use the invention, i.e., failing to provide an enabling disclosure. The Examiner's grounds for this rejection are addressed below.

A. Applicants teach uses of the claimed methods which are credible on their face to those of ordinary skill in the art

The Examiner states that "[I]n vitro and animal model studies have not correlated well with in vivo clinical trial results in patients" and that "drugs such as costimulatory-based biopharmaceutical drugs can be species-and model-dependent." Applicants respectfully traverse these statements.

Applicants direct the Examiner's attention to the guidelines for examination of applications for compliance with 35 U.S.C. § 101, which provide that "[i]f an applicant has asserted that the claimed invention is useful for any particular purpose and that assertion would be considered credible by a person of ordinary skill in the art, the Examiner should not impose a rejection based on §101". If the asserted utility is credible, there is no basis for an Examiner to challenge such a claim on the grounds that it lacks utility under § 101. Although the Examiner has rejected the claims under § 112, first paragraph for failing to teach how to use B7-2 fusion

proteins *in vivo*, it is Applicants' position that the principles of the new guidelines for §101 rejections apply equally to §112, first paragraph rejections. Applicants position is supported by the CAFC which stated in *In re Brana,* 34 USPQ 2d 1436 (Fed. Cir. 1995); quoting *In re Marzocchi* 58 C.C.P.A. 1069, 439 F.2D 220, 223, 169 U.S.P.Q. 367, 369 (CCPA 1971))

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of §112 ***unless there is reason to doubt the objective truth of the statements*** contained therein which must be relied on for enabling support. (Emphasis added).

In view of the above, the Court concluded that:

> [T]he PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure...only ***after the PTO provides evidence*** showing that one of ordinary skill in the art would ***reasonably doubt the asserted utility*** does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. (Emphasis added).

In view of these guidelines and statements by the CAFC, it is clear that the Examiner has failed to provide evidence establishing that one of ordinary skill in the art would reasonably doubt the asserted utility. At most, the Examiner doubts "the relative efficacy" of the clamed methods.

Throughout the specification, Applicants clearly teach that B7-2 costimulates T cell activation and that the absence of B7-2 costimulation can result in inhibition of T cell activation and establishment of tolerance. As set forth, e.g., at page 7, line 9-27, page 38, lines 13-15, page 38, lines 28-33 of the specification, Applicants teach that agents that modulate the interaction between B7-2 and its natural ligand can be used for inducing both general immunosuppression and antigen-specific tolerance in a subject by, for example, blocking the functional interaction of a B7 molecule with its receptor, e.g., CD28 or CTLA4, to thereby block co-stimulation through the receptor-ligand pair. Inhibition of T cell responses and induction of immunosuppression or T cell tolerance according to the methods described herein may be useful prophylactically, in

preventing transplantation rejection (solid organ, skin and bone marrow) and graft versus host disease, especially in allogeneic bone marrow transplantation. The methods of the invention may also be useful therapeutically, in the treatment of autoimmune diseases, allergy and allergic reactions, transplantation rejection, and established graft versus host disease in a subject.

Applicants teach a number of agents within the scope of the claims. For instance, a soluble form of B7-2 (page 39, line 23-25), an antibody that recognizes B7-2 (page 34, line 9-11), soluble forms of B7-2 receptors such as CTLA4Ig and CD28Ig (page 7, line 19-20). Accordingly, the specification teaches various agents which can be used in the claimed methods.

Applicants also teach a variety of *in vivo* uses for the claimed method of inhibiting the interaction between B7-2 and its natural ligand. These uses are credible to a person of skill in the art on their face. It is not necessary for Applicants to provide actual *in vivo* data as to whether the method of the instant invention can be used for the treatment of human disease, but rather Applicants must adequately teach the ordinarily skilled artisan how to make or use the claimed invention. In *Cross v. Iizuka* (753 F.2d 1040 (Fed. Cir. 1985) the court established that the enablement requirement's how-to-use aspect is met when pharmacological activity in an *in vitro* environment is demonstrated and, accordingly, that the ordinarily skilled artisan can determine dosage levels for therapeutic administration without undue experimentation.

Moreover, a soluble form of a B7-2 ligand, CTLA4Ig, has been used successfully, e.g., for preventing organ transplant rejection and graft versus host disease in animal models (Lenshow et al. (1992) *Science* 257:789; Lin et al. (1993) *J. Exp. Med.* 178:1801; Blazar et al. (1994) *Blood*, 83: 3815, copies of which are submitted herewith as Appendices B-D, respectively). This molecule has also been used successfully to establish immunosuppression *in vivo* (Linsley et al. (1992) *Science* 257:792, a copy of which is attached hereto as Appendix E). In addition, antibody to B7-2 have been found to be as effective as CTLA4Ig in preventing rejection of allografts *in vivo*, with antibody to B7-2 in combination with antibody to B7-1 being even more effective (Lenschow et al. (1995) *Transplantation* 60:1171, attached hereto as Appendix F).

Thus, in view of Applicants' teachings, a person of skill in the art would find the proposed *in vivo* uses for the claimed methods credible on their face. Accordingly, the Examiner has failed to establish a *prima facie* case of lack of enablement for the claimed methods.

With respect to the various references relied upon by the Examiner in support of his position, Applicants would like to make the following remarks of record.

The Examiner relies on the teachings of Kahan as supporting his assertion that "[p]harmaceutical data in the absence of *in vivo* clinical data are unpredictable. In support of this statement, the Examiner quotes Kahan as teaching that "no *in vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficacy" and that "there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from *in vitro* systems to *in vivo* conditions."

In response to the Examiner's reference to the teachings of Kahan et al., Applicants point out that the comments in the Kahan reference are not specific to the claimed methods and, in fact, no discussion of therapies that block costimulation is included in the reference. In addition, the statement quoted by the Examiner is made in reference to human clinical trials, (see page 558, the last paragraph column 1 and the first paragraph column 2, under the heading "Immunosuppressive drug trials") and does not refer to whether the use of immunosuppressive agents in vivo is credible, clearly immunosuppressives are being used clinically to treat human patients, but to predicting efficacy and dosage based on in vitro experiments. In relying on the teachings of Kahan et al., the Examiner seems to be implying that without data from human clinical trials, the Patent Office will not consider methods claims which can be applied to therapeutic treatment to be enabled. This is an unreasonable requirement since in no case has a Federal court required an applicant to support an asserted utility with data from human clinical trials.

As further support of the rejection, the Examiner relies on Blazar et al. as evidence of the unpredictability of the art with respect to pharmaceutical therapies in the absence of clinical data, stating:

> issues such as tissue distribution, half-life, affinitiy and avidity obtained with these various CD28-B7-specific reagents might prove to be highly important in achieving

GVHD protection (and that) any conclusion regarding the efficacy of CD28/B7 blockade on altering *in vivo* immune response should be interpreted in light of the type of reagent infused

Applicants point out that although Blazar et al. teaches that different agents may have different tissue distribution, half life, affinity, and avidity, the development of a dosage regimen appropriate for agents that inhibit a costimulatory signal in a T cell is well within the ability of one of average skill in the art, and that such routine determinations do not constitute undue experimentation.

The Examiner further relies on the disclosure of Blazar et al. as indicating that the methods as claimed are not effective therapeutics, stating that "anti-CD80 or anti-CD86 antibodies were ineffective in preventing T cell CDC8-mediated GVHD lethality; that each antibody was partially effective in CD4-mediated GVHD lethality and that the combination of anti-CD80 and anti-CD86 antibodies were effective in preventing GVHD lethality in murine experimental models."

In response, Applicants point out that, contrary to the Examiner's interpretations of the disclosure of Blazar et al., the teachings of Blazar et al. are in fact supportive of the immediate claims of the invention. Blazar et al. discloses data that suggests administration of antibodies to B7-1 (also known as CD80) or antibodies to B7-2 (also known as CD86) are at least partially effective therapeutics in the treatment of GVHD. For example, antibodies to B7-1 or B7-2 administered individually to bm12 (MHC class II disparate) recipient mice conferred a 37% actuarial survival rate (page 3252, column 1, second paragraph, and Figure 3A), and reduced weight loss (Figure 3B) in an *in vivo* animal model of GVHD, compared to negative control mice. Antibodies to B7-1 or B7-2 administered individually to bm1 (MHC class I disparate) recipient mice also conferred a recorded advantage in the treatment of the animal GVHD model in the areas of weight loss and hematocrit levels, indicating a therapeutic effect (page 3252, column 1, second paragraph, and Figure 4B). The fact that in the assay system of Blazar et al., administration of both antibodies to B7-1 and antibodies to B7-2 conferred optimal anti-GVHD effects, does not negate the benefit of the administration of one of the antibodies (e.g., an antibody to B7-2).

The Examiner relies on the disclosure of Perrin et al. in further support of his assertion that the present invention is not enabled by the specification. The Examiner claims that Perrin et al. discloses that administration of antibodies to B7-1 attenuated the first clinical disease episode but not the relapse in an (autoimmune) experimental allergic encephalomyelitis (EAE) mouse model system. The Examiner also states that Perrin et al. further discloses that administration of antibodies to B7-2 had no significant effect on the course of disease, and that the administration of both antibodies to B7-1 and B7-2 resulted in the exacerbation of disease in the animal model system.

Applicants first note that the disclosure of Perrin et al. does not indicate that antibodies to B7-2 are not effective at inhibiting the interaction of B7-2 with its natural ligand on the surface of an immune cell when the antibodies are contacted to the immune cell, as presently claimed. Furthermore, the disclosure of Perrin et al. does not indicate that other such agents set forth in the application (non-elected species) are not effective at inhibiting the interaction of B7-2 with its natural ligand on the surface of an immune cell when the agents are contacted to the immune cell, as presently claimed.

Applicants disagree with the Examiner's assertion that the disclosure of Perrin et al. indicates that the methods as claimed are not effective as therapeutics. In fact, the disclosure of Perrin et al. presents evidence that administration of a CTLA4-Ig fusion protein attenuates disease in the model system. This evidence ***supports*** Applicants' position that CTLA4-Ig fusion protein (a non-elected species of the agent) inhibits the interaction of B7-2 with its natural ligand on the surface of an immune cell when contacted to the immune cell. Regarding the Examiner's assertions about the lack of efficacy of antibodies to B7-1 and B7-2 in attenuating disease in the model system of Perrin et al., Applicants note the significant limitations of the study reported in Perrin et al. First, the study is limited with respect to the dosage and regimen of administration of the antibodies to treat disease. Only a ***single dosage*** was tried, and only a ***single administration*** was performed. This limitation is at least partially acknowledged by Perrin et al. which teaches that the lack of therapeutic effects resulting from administration of antibodies to B7-1 and B7-2 "may reflect that the effect of single dose anti-CD80 [anti-B7-1] and [anti-CD86 anti-B7-2] treatment is transient, and that continual administration of the antibodies would be

required to obtain a lasting effect." Second, the model system used in the experiments of Perrin et al. requires immunization with myelin basic protien accompanied by an injection of pertussis toxin (PT) to produce the disease. Such a model system for disease is susceptible to interactions of the administered antibodies with the agents used to induce disease, causing artifactual results. This potential flaw in the model is acknowledged by the disclosure of Perrin et al., which states:

> it is possible that an interaction between the anti-B7 reagents and PT has resulted in disease exacerbation.

Perrin et al. goes on to indicate that the findings in the disclosure are actually contradictory to their own earlier findings in another model system that does not utilize the same agents for disease induction, stating

> In an active model of EAE which does not require PT treatment, injection of anti-CD80 [anti-B7-1] plus anti-CD86 [anti-B7-2] on day 2 post-immunization resulted in decreased incidence and severity of clinical disease rather than the exacerbated disease observed in the present study (Racke et al., 1995).

Given the limitations of the study reported in the disclosure of Perrin et al., the results reported therein cannot reasonably be said to indicate a lack of enablement of anti-B7-2 and/or anti-B7-1 antibodies in in vivo therapy, in light of the availability of other disclosures which indicate an efficacy of anti-B7-2 and/or anti-B7-1 antibodies. Such disclosures include Lenschow et al., (*J. Exp. Med. 181*: 1145-1155 (1995)) which teaches that administration of antibodies to B7-2 was protective of disease in the NOD mouse model of autoimmune diabetes, and Racke et al., (*J. Clin. Invest. 96*: 2195-2203 (1995)) cited by Perrin et al. in the above quoted passage (for convenience, both Lenschow et al. and Racke et al. are attached as Appendices G and H).

The Examiner cites the disclosure of Yi-qun et al. as further evidence that the methods as claimed are not effective as therapeutics. More specifically the Examiner states that the disclosure of Yi-qun et al. indicates that: a) inhibition of a T cell response to soluble antigens will require the blocking of both B7-2 and B7-1 to be effective; and also that: b) it is unlikely that ongoing T cell response will be susceptible to inhibition by anti-B7 reagents, for example in autoimmune disease. Applicants respectfully disagree with the Examiner's interpretation of Yi-

qun et al. as indicating that the methods as claimed are not effective as therapeutics, and more specifically with the Examiner's interpretation of Yi-qun et al. as indicating that inhibition of a T cell response to soluble antigens will require the blocking of both B7-2 and B7-1 to be effective. In fact, the disclosure of Yi-qun et al. supports Applicants' position that blocking of the B7-2 response is sufficient to produce a therapeutic effect by teaching that blocking of either B7-1 or B7-2 independently, produces an inhibitory effect on memory T cell stimulation (see for instance Figure 3B). Although Yi-qun et al. teach that blocking both B7-1 and B7-2 produces a stronger effect on memory T cell inhibition than blocking one or the other of B7-1 and B7-2 co-stimulation, such an occurrence does not negate the utility of blocking one or the other response in conjunction with administration of an agent which promotes hemostasis. Furthermore, Applicants point out that the disclosure of Yi-qun et al. acknowledges the limitations of the findings reported therein, when it states:

> These data do not exclude the possibility that in certain *in vivo* circumstances B7-1 is of equal or dominant importance. Differences in the availability of B7-1 and B7-2 for co-stimulation under different conditions of antigen presentation might determine their relative importance *in vivo*.

Therefore, it is inaccurate to determine from the disclosure of Yi-qun et al. that both B7-1 and B7-2 must be blocked to produce a therapeutic effect and it is inaccurate to determine that blockade of a costimulatory signal in a T cell, as recited in the present methods, is insufficient to produce a therapeutic effect.

The Examiner also asserts that the (non-elected) species of a soluble form of B7-2, as an agent which inhibits the interaction of B7-2 with its natural ligand on the surface of an immune cell is not enabled by the instant specification because it does not function (to inhibit the interaction of B7-2 with its natural ligand) in a manner consistent with the instant disclosure. The Examiner turns to Sturmhoefel et al., which teaches that soluble B7 molecules can be immunostimulatory, in support of this contention.

Applicants traverse these comments and point out that the instant specification teaches that agents that modulate the interaction between B7-2 and its natural ligand can also be used for

upregulating immune responses by delivery of a costimulatory signal to T cells. For example, the specification teaches that a stimulatory form of a B7-2 fusion protein can be used to stimulate an immune response. At page 7, line 29-33, and page 38, line 36 through page 39, line 2 of the specification, Applicants teach that delivery of a stimulatory form of B7-2 can be used to upregulate an immune response. Thus, the teachings of Sturmhoefel are not inconsistent with Applicants' teachings. Moreover, the pending claims, although they require the inhibition of the interaction between B7-2 and its natural ligand, do not require that the immune response be modulated in a specific direction, e.g., up or down. Applicants point out that certain agents that inhibit the interaction between B7-2 and its natural ligand may be capable of sending a signal via a costimulatory receptor on a T cell (e.g., stimulatory forms of soluble B7-2 may transmit a signal via CD28) while other agents that inhibit the interaction between B7-2 and its natural ligand may not be capable of sending such a signal (e.g., anti-B7-2 antibodies).

The Examiner further asserts that it is not clear that there is objective evidence that supports the ability of CD28Ig, any agent that blocks the interaction of B7-1 with its natural ligand, and/or antibody that binds a cytokine, would be immunosuppressive in the targeted conditions encompassed by the claimed methods. In support of this assertion, he cites Debets et al. as disclosing the "potential therapeutic uses of cytokine antagonists, such as antibodies, including the importance of testing appropriate models and the limitations of double-edged sword of such antagonists."

Applicants traverse these remarks, and point out that the disclosure of Debets et al. fails to support the Examiner's assertion. First, the disclosure of Debets et al. does not discuss costimulation. Debets et al. is a review of cytokine antagonists and their potential therapeutic uses. Debets et al. points out the potential agonizing effects of anti-cytokine therapy and cautions that "the therapeutic use of intravenous Ig preparations, displaying binding capacities for IL-1α and IL-6, should be considered carefully." However, Debets et al. does not state or imply that these potential agonizing effects are prohibitive to therapy, nor does it teach or suggest that the present claims are not enabled.

The Examiner further states that the specification does not identify the appropriate cytokine targets and the appropriate conditions to be targeted by anti-cytokine antibodies in

combination with the B7-2 specific antagonists. However, it is Applicants' position that the determination of the appropriate cytokine targets and the appropriate conditions to be targeted are within the ability of one of ordinary skill in the art through no more than routine experimentation. The specification teaches the use of blocking antibodies to cytokines which function as immunomodulating agents (page 39, line 12-14), teaches specific blocking antibodies against IL-9 and IL-12 (page 47, line 29), and further teaches assays for the identification of cytokines induced by costimulation, which prevent tolerance (page 47, line 17- page 48, line 12). This guidance, coupled with that which is known in the art, enables the skilled practitioner to make and use the invention as claimed.

Applicants have identified the B7-2 molecule as a ligand for CTLA4 and CD28, they provide experimental evidence that B7-2 plays an active role in costimulation, and further provide methods for blocking B7-2 costimulation, as well as guidance in the therapeutic treatment of disease by blocking the interaction of B7-2 with its natural ligands. The Examiner, has failed a *prima facie* case of lack of enablement for the claimed methods.

B. Enablement for B7-2, CD28, CTLA4, Ligand and Agents

The Examiner further rejects claims 28, 66, 67, 70 and 71, stating that the specification does not reasonably provide enablement for any B7-2, CD28, CTLA-4, ligand, or agent. More specifically, the Examiner states:

> Applicant has not provided sufficient biochemical information (e.g. molecule weight, amino acid composition, N-terminal sequence, etc.) that distinctly identifies the B7-2, CD28, CTLA4, ligands, agents other than those encompassed by the disclosure of the particular human/murine/mouse costimulatory molecules disclosed in the specification as filed. B7-2, CD28, CTLA4, ligand or agent may have some notion of the activity of the receptor, ligand or agent, claiming biochemical molecules by a particular name given to the protein (e.g. receptor or ligand) by various workers in the field fails to distinctly claim what the protein is and what the compositions are made up of.

In support of this assertion, the Examiner cites Coyle et al., as disclosing that B7-1 and B7-2 exhibit pronounced differences in structural and functional characteristics and also disclose the increasing complexity in costimulatory signal regulating T cell function, and that a number of

molecules are poorly understood and likely have distinct roles in the regulation of T cells. The Examiner further cites Harlan as teaching that it is a long way to product, Ngo et al. as teaching that there is no well understood and predictable relationship between the sequence of a peptide and its tertiary structure (or its activity), and Skolnick et al. as teaching that assigning functional activities for any particular protein or protein family based upon sequence homology is inaccurate. The Examiner further cites Lederman et al. as disclosing that a single amino acid substitution in a common allele ablates binding of a monoclonal antibody. The Examiner further cites Li et al., as teaching the dissociation of immunoreactivity from other biological activities when constructing analogs.

This rejection is respectfully traversed, on the grounds that Applicants have described a representative number of species of agents which inhibit B7-2 binding with its natural ligand (e.g., B7-2 molecules, CD28 molecules, CTLA-4 molecules, B7-2 antibodies) to enable the claimed invention. Applicants have described the encoding nucleotide sequence and the amino acid sequence of both mouse and human B7-2 molecules. The encoding nucleotide sequence and amino acid sequence of at least mouse and human CD28 and CTLA-4 molecules are known in the art, both of which are ligands of B7-2. Applicants have further described several agents which inhibit B7-2 binding with its natural ligand (which are known in the art) and also have provided guidance in the identification of such additional agents. The identification of additional CD28 molecules, CTLA-4 molecules, or additional ligands of B7-2, and also additional agents which inhibit B7-2 binding with its natural ligand is within the ability of one of ordinary skill in the art through routine experimentation, e.g., through routine screening.

The disclosure of Coyle et al., cited by the Examiner supports Applicants position, that the identification of other B7-2 molecules is accomplished through routine analysis of nucleotide and amino acid sequence, as it indicates that B7-2 molecules are sufficiently distinct from other B7 family members to be identifiable and readily categorized appropriately as B7-2 molecules. Additional B7-2 molecules (e.g., orthologs) can be readily identified as such by their encoding nucleic acid and amino acid sequence via hybridization assays or cross-reactivity with antibodies to the known B7-2 molecules. Once identified, the identify of a B7-2 molecule can be confirmed via phylogenic analysis of the B7-2 sequence (nucleic acid or amino acid) in comparison to the

B7 superfamily, as indicated by Coyle et al. which discloses that B7-2 molecules are readily distinguished from related B7 family members (e.g., the closest family member, B7-1, exhibits pronounced differences in structural and functional characteristics from B7-2, as per Coyle et al.). Furthermore, Applicants provide guidance in Example 7 in the form of identification of a critical region of B7-2 which delineates the variable domain of B7-2 as necessary for ligand binding, which will aid in such an analysis.

With respect to the enablement of natural ligands of B7-2, Applicants provide experimental evidence that CTLA4 and CD28 function as natural ligands for B7-2. The nucleic acid and amino acid sequences of mouse and human CTLA4 and CD28 were known in the art at the time of filing (Dariavach, et al. (1988) *Eur. J. Immunol.* 18(12), 1901-1905; Brunet, J.F., et al. (1987) *supra*; Brunet, J.F. et al. (1988) *Immunol. Rev.* 103:21-36; and Freeman, G.J., et al. (1992) *J. Immunol.* 149, 3795-3801, Aruffo and Seed, Proc. Natl. Acad. Sci. 84:8573-8577 (1987)). This sequence information allows the identification of CTLA4 or CD28 orthologs or variants through routine experimentation. The identification of additional ligands for B7-2 is within the ability of one of ordinary skill in the art through routine experimentation, e.g. through routine functional screening and sequence analysis.

The Harlan et al. reference teaches the need for an appropriate clinical setting for evaluation of clinical therapeutics. This reference fails to teach or suggest that agents exemplified by Applicants will not work. In addition, Applicants reiterate their previous statement that in no case has a Federal court required an applicant to support an asserted utility with data from human clinical trials.

In response to the Examiner's citation of Ngo et al. as disclosing that there is no well understood or predictable relationship between the sequence of a peptide and its tertiary structure, Applicants point out that functional assays for a ligand of B7-2 and also for an agent which inhibits B7-2 binding with its natural ligand are available to one of ordinary skill in the art, and that the performance of such assays constitutes routine experimentation.

In response to the Examiner's citation of Skolnick et al., as teaching that assigning functional activities for a particular protein or protein family based upon sequence homology is inaccurate, Applicants point out that the Skolnick et al. disclosure is concerned with the

identification of function of a protein only from sequence data, and does not readily apply to the identification of the function of molecules for which functional assays have been developed and are readily available. The availability of such functional assays for a B7-2 molecule (the ability to bind B7-2 receptors, and also the ability to activate B7-2 receptors), for ligands of the B7-2 molecules (the ability to bind B7-2, and also the ability to be activated by B7-2 receptors), and for agents which inhibit B7-2 binding with its natural ligand (binding assays and also inhibition in costimulation assays) coupled with information available through sequence analysis allows one of ordinary skill in the art to practice the claimed invention with no more than routine experimentation.

With respect to the Examiner's citation of the disclosure of Lenderman et al., and Li et al., it is Applicants' position that the fact that a single amino acid substitution in a common allele may ablate binding of an antibody, as disclosed by Lenderman et al., or that a given analog may not be recognized by an antibody to its predecessor is of no consequence. First, Applicants point out that such a single amino acid substitution may ablate binding of one or a few antibodies to a given molecule, but rarely does a sequence change result in the ablation of binding of all antibodies which bind the original molecule, in that a subset of antigens is conserved. Furthermore, the generation of antibodies to a given molecule is routine and predictable, and thus, both monoclonal and polyclonal antibodies to an allele or analog can be readily generated by the skilled practitioner.

Rejection of Claims 28, 66, 67, 70, and 71 Under 35 U.S.C. § 112, First Paragraph as Not In the Possession of Applicants

Claims 28, 66, 67, 70, and 71 have been rejected under 36 U.S.C. 112, first paragraph as drawn to an invention which Applicants were not in possession of at the time of filing. In his rejection, the Examiner first asserts a lack of possession of B7-2 by Applicants. The Examiner states that Applicants are relying on biological activities and the disclosure of a limited number of species to support the entire genus of B7-2 molecules, and of natural ligands for B7-2 molecules. The Examiner asserts that "there is insufficient guidance based on the reliance of B7-2 set forth in SEQ ID NO: 2 and 23 to direct a person of skill in the art to select or to predict

particular sequences as essential for identifying B7-2 molecules encompassed by the claimed specificities." In support of his assertions, he cites Coyle et al. (*Nature Immunology 2*: 203-209, 2001) as teaching the structural and functional differences in B7-1 and B7-2, and the increasing complexity in costimulatory signal regulating T cell function.

Applicants respectfully traverse this rejection. According to the "Written Description Requirement":

> The written description requirement for a claimed genus may be satisfied through sufficient description of ***a representative number of species*** by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, ***or by a combination of such identifying characteristics sufficient to show that applicant was in possession of the claimed genus***. ... a representative number depends upon whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed.... Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces. (emphasis added)

The number of species of B7-2 molecules identified by Applicants (human and mouse) are sufficient to show that Applicant was in the possession of the claimed genus. This is supported by the disclosure of Coyle et al., cited by the Examiner, which discloses that B7-2 molecules are readily distinguished from related B7 family members (e.g., the closest family member being, B7-1). The disclosure of Coyle et al. fails to support the assertion of the Examiner, indicating that other B7-2 molecules are readily identified as such, as opposed to confused with other related B7 family members.

The Examiner similarly asserts a lack of possession by Applicants of the natural ligands of B7-2, stating that "a person of skill in the art would not know which sequences are essential, which sequences are non-essential, and what particular sequence lengths identify essential sequences for identifying a B7-2 molecule and/or a natural ligand thereto, encompassed by the claimed invention." Applicants disagree with this statement, and respectfully traverse this aspect of the rejection, arguing that a representative number of species of natural ligands of B7-2 are presented in the application and known in the art. Applicants provide far more than a mere

statement that the natural ligand of B7-2 is part of the invention and reference to a potential method for isolating it. Applicants provide experimental evidence that CTLA4 and CD28 function as natural ligands for B7-2. The nucleic acid and amino acid sequences of mouse and human CTLA4 and CD28 were known in the art at the time of filing (Dariavach, et al. (1988) *Eur. J. Immunol.* 18(12), 1901-1905; Brunet, J.F., et al. (1987) *supra*; Brunet, J.F. et al. (1988) *Immunol. Rev.* 103:21-36; and Freeman, G.J., et al. (1992) *J. Immunol.* 149, 3795-3801, Aruffo and Seed, Proc. Natl. Acad. Sci. 84:8573-8577 (1987)). This sequence information, coupled with function, is sufficient to indicate to one of skill in the art would that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus of ligands of B7-2.

The Examiner also asserts a lack of possession of the antibody reactive with CD28, CTLA4, and cytokine, as well as an agent that blocks the interaction of B7-1 with its natural ligand, encompassed by the claimed methods. More specifically the Examiner states:

> "there is insufficient guidance based on the reliance of a limited disclosure to direct a person of skill in the art to select or to predict particular sequences as essential for identifying any "CD28", "CTLA-4", "cytokine" specificity or "agent", encompassed by the claimed invention. Mere idea or function is insufficient for written description; isolation and characterization at a minimum are required."

In support, the Examiner further refers to the disclosure of Lenderman et al. *(Molecular Immunology 28*: 1171-1181, 1991) as disclosing that a single amino acid substitution in a common allele ablates binding of a monoclonal antibody, and also to the disclosure of Li et al. (*PNAS 77*: 3211-3214, 1980) as disclosing that dissociation of immunoreactivity from other biological activities when constructing analogs. In traversal, Applicants submit that the present application does far more than put forth the "mere idea or function" of an antibody to CD28, an antibody to CTLA-4, an antibody to a cytokine, as well as an agent, as encompassed by the claimed invention. A variety of such antibodies and agents were known in the art at the time of filing, and this variety constitutes a representative number of species. The application is not required to teach what is already known. The fact that recognition of a given antigen by an

antibody can be ablated by a change in the antigen sequence is irrelevant, as production of antibodies to a given antigen is common and routine, and as stated in the above quoted passage of the written description requirement guidelines, description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces.

Thus, in view of all of the above, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of Claims 28, 66, 67, 70, and 71 under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 28, 66, 67, and 70-71 Under 35 USC, 112, Second Paragraph

Claims 28, 66, 67, and 70-71 have been rejected under 35 USC, 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. More specifically, the Examiner states that the claims:

> are [in]definite in its recitation of "inhibiting the interaction ... with its natural ligand" because the nature of the "interaction" and the "ligand" is ambiguous. Therefore the metes and bounds of the claimed methods encompassing inhibiting interactions and ligands are not readily apparent.

Applicants respectfully traverse this rejection and point out that the claims specifically state that the agent contacted inhibits B7-2 binding with its natural ligand, which clearly indicates the nature of the interaction which is inhibited to one of ordinary skill in the art.

The Examiner further states:

> The claims are indefinite in the recitation of "ligand" in that they only describe the products of interest by an arbitrary protein name.

Applicants respectfully traverse this rejection and argue that the claims state "a natural ligand on the surface of an immune cell." This clearly refers to ligands found in nature expressed on the surface of an immune cell. One of skill in the art would readily understand the metes and bounds of the invention as it relates to this term, with respect to structure and function, especially in light of the fact that two such ligands for B7-2 are taught in the specification, i.e., CTLA4 and CD28, and orthologs in different species of these two ligands are known in the art.

Rejection of Claims 28, 66, 67, 70-71 Under 35 U.S.C. §102(e)

Claims 28, 66, 67, 70-71 have been rejected under 35 U.S.C. §102(e) as being anticipated by the disclosure of deBoer et al., (U.S. Patent No.5,747,034 (1998)). More specifically the Examiner states:

> deBoer et al., teach methods of inhibiting immune responses both in vitro and in vivo with combinations of B7-specific inhibitors, including the use of both B7-1-specific and B7-2-specific antibodies. . . .The claimed functional limitations would be inherent properties of the referenced methods of inhibiting immune responses with B7-specific antibodies.

This rejection is respectfully traversed. First, Applicants point out that the priority date for the present claims is at least as early as USSN 08/101,624, filed July 26, 1993, to which the present application claims priority. Support for this earlier priority date is discussed above in detail under the heading of "Filing Date of the Instant Claims." The priority document USSN 08/101,624 provides specific support for the use of an agent to inhibit B7-2 binding with its natural ligand. The filing date of the deBoer et al. application is February 18, 1994, after Applicants' priority date. Because the priority date of the present application is prior to the filing date of the deBoer et al. patent, the deBoer et al. patent is not available prior art for use against the present claims under 35 USC §102(e).

In conclusion, the disclosure of de Boer et al. does not anticipate Claims 28, 66, 67, 70-71, since the disclosure is not available prior art.

Rejection of Claims 28, 66, 67, and 70-71 Under 35 U.S.C. §103(a)

Claims 28, 66, 67, and 70-71 have been rejected under 35 U.S.C. §103(a) as being obvious over deBoer et al. in view of Linsley et al. (U.S. Patent No. 6,090,914 (2000)). More specifically, the Examiner states:

> Given the teachings of deBoer et al. and Linsley et al.; the ordinary artisan would have been motivated to target both B7-1 and B7-2 with B7-specific antibodies in order to achieve a higher efficacy in inhibiting immune responses in vitro and in vivo, given the

contribution of both the B:T cell interactions as well as observations and/or indications that CTLA4Ig achieve its efficacy by inhibiting both B7-1 and B7-2.

This rejection is respectfully traversed. Applicants first point out that only one of the rejected claims (Claim 67) is specifically directed to blocking the interaction of both B7-2 and B7-1 with their natural ligands.

Applicants next reiterate that the priority date for the present claims is at least as early as USSN 08/101,624, filed July 26, 1993, to which the present application claims priority. Support for this earlier priority date is discussed above in detail under the heading of "Filing Date of the Instant Claims". The priority document of USSN 08/101,624 provides specific support for the use of an agent to inhibit B7-2 binding with its natural ligand. The filing date of the deBoer et al. application is February 18, 1994, and the filing date of the Linsley et al. application is April 15, 1994. Because the priority date of the present application is prior to the filing date of these patents, neither the deBoer et al. patent nor the Linsley et al. patent are available prior art for use against the present claims under 35 USC §102(e).

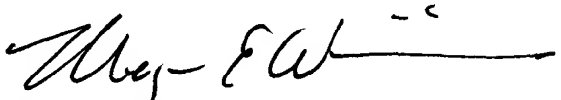
SUMMARY

In view of the foregoing remarks, reconsideration of the rejections and allowance of all pending claims is respectfully requested.

If a telephone conversation with Applicants' attorney would expedite the prosecution of the above-identified application, the examiner is urged to call Applicants' attorney at (617) 227-7400.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP



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Dated: February 25, 2002

APPENDIX A

Version With Marking to Show Changes Made to the Specification and the Abstract of the Disclosure

Changes made to the Title on the first page:

[NOVEL CTLA4/CD28 LIGANDS AND USES THEREFORE]

METHODS FOR INHIBITING THE INTERACTION OF B7-2 WITH ITS NATURAL LIGAND

Changes made to the first page, paragraph under "Related Applications":

This application is a continuation application of U.S. Serial No. 08/479,744, filed on June 5, 1995, now U.S. Patent 6,084,067, which is a continuation-in-part of U.S. Serial No. 08/280,757, entitled "Novel CTLA4/CD28 Ligands and Uses Therefor" filed July 26, 1994, now U.S. Patent No. 6,130,316, which is a continuation-in-part of U.S. Serial No. 08/109,393, entitled "Novel CTLA4/CD28 Ligands and Uses Therefor" filed August 19, 1993, pending, which is a continuation-in-part of U.S. Serial No. 08/101,624, also entitled "Novel CTLA4/CD28 Ligands and Uses Therefor", filed July 26, 1993, now U.S. Patent No. 5,942,607. U.S. Serial No. 08/479,744, now U.S. Patent No. 6,084,067, is also a continuation-in-part of U.S. Serial No. 08/147,773, entitled "Tumor Cells Modified To Express B7-2 And B7-3 With Increased Immunogenicity And Uses Therefor" filed November 3, 1993, now abandoned. The contents of each of these applications is incorporated herein by reference.

Changes made to title in the Abstract of the Disclosure:

[NOVEL CTLA4/CD28 LIGANDS AND USES THEREFORE]

METHODS FOR INHIBITING THE INTERACTION OF B7-2 WITH ITS NATURAL LIGAND

Changes made to the Abstract of the Disclosure:

[Nucleic acids encoding novel CTLA4/CD28 ligands which costimulate T cell activation are disclosed. In one embodiment, the nucleic acid has a sequence which encodes a B lymphocyte antigen, B7-2. Preferably, the nucleic acid is a DNA molecule comprising at least a portion of a nucleotide sequence shown in Figure 8, SEQ ID NO:1 or Figure 14, SEQ ID NO:23. The nucleic acid sequences of the invention can be integrated into various expression vectors, which in turn direct the synthesis of the corresponding proteins or peptides in a variety of hosts, particularly eukaryotic cells, such as mammalian and insect cell culture. Also disclosed are host cells transformed to produce proteins or peptides encoded by the nucleic acid sequences of the invention and isolated proteins and peptides which comprise at least a portion of a novel B lymphocyte antigen. Proteins and peptides described herein can be administered to subjects to enhance or suppress T cell-mediated immune responses.]

The present invention relates to, inter alia, methods for inhibiting the interaction of the B-lymphocyte antigen, B7-2, with its natural ligand on the surface of an immune cell are disclosed. The methods comprise contacting the immune cell with an agent which inhibits B7-2 binding with its natural ligand, to thereby inhibit the interaction. Examples of such agents are provided, and include a soluble form of B7-2, an antibody that recognized B7-2. The method may also include contacting the immune cell with an agent that blocks the interaction of B7-1 with its natural ligand. Further, the method may include contacting the immune cell with an immunomodulating agent, for example, an antibody reactive with CD28, an antibody reactive with CTLA4, an antibody reactive with a cytokine, a CTLA4Ig fusion protein, a CD28Ig fusion protein, and an immunosuppressive drug. Both *in vivo* and *in vitro* applications of the method are disclosed.

(Academic Press, New York, 1982), vol. 4B, chap. 36.
2. B. Schaeffer and K. S. Thomson, in *Aspects of Vertebrate History*, L. L. Jacobs, Ed. (Museum of Northern Arizona Press, Flagstaff, AZ, 1980), pp. 19–33.
3. D. W. Yalden, *Zool. J. Linn. Soc.* **84**, 291 (1985).
4. A. S. Romer, *Vertebrate Paleontology* (Univ. of Chicago Press, Chicago, ed. 3, 1966); E. Jarvik, *Basic Structure and Evolution of Vertebrates* (Academic Press, New York, 1980), vol. 2.
5. P. L. Forey, *J. Vertebr. Paleontol.* **4**, 330 (1984).
6. S. Løvtrup, *The Phylogeny of the Vertebrata* (Wiley, New York, 1977).
7. J. G. Maisey, *Cladistics* **2**, 201 (1986).
8. P. Janvier, *J. Vertebr. Paleontol.* **1**, 121 (1981).
9. M. Goodman, M. M. Miyamoto, J. Czelusniak, in *Molecules and Morphology in Evolution: Conflict or Compromise?*, C. Patterson, Ed. (Cambridge Univ. Press, New York, 1987), pp. 141–176; M. Goodman *et al.*, *J. Mol. Evol.* **27**, 236 (1988). The trees in these analyses depict a monophyletic Cyclostomata but differ in whether the hemoglobin-myoglobin duplication occurred before or after the divergence of cyclostomes and gnathostomes. The trees are rooted with the relatively distant outgroups of mollusks and arthropods because of the sporadic taxonomic distribution of globins in invertebrates. A recent comparison of Mn-Fe superoxide dismutases [M. W. Smith and R. F. Doolittle, *J. Mol. Evol.* **34**, 175 (1992)] included 432 nucleotides from a lamprey, a hagfish, and a cephalochordate. Trees produced by parsimony and distance analyses depicted sister-group relationships between hagfishes and gnathostomes and between lampreys and hagfishes, respectively. In both cases the common branch uniting two of the taxa was short; this geometry suggests that neither result is strongly supported.
10. A. C. Wilson, S. S. Carlson, T. J. White, *Annu. Rev. Biochem.* **46**, 573 (1977).
11. The oldest gnathostomes are acanthodians [R. Denison, in *Handbook of Paleoichthyology*, H.-P. Schultze, Ed. (Fischer-Verlag, New York, 1979), vol. 5] from the Lower Silurian, whereas unequivocal lamprey and hagfish fossils are known from the Mississippian [P. Janvier and R. Lund, *J. Vertebr. Paleontol.* **2**, 407 (1983)] and the Pennsylvanian [D. Bardack, *Science* **254**, 701 (1991)], respectively. The oldest fossil agnathans are from the Upper Cambrian [J. E. Repetski, *Science* **200**, 529 (1978)]. Depending on the relationships of fossil and living taxa (*5, 8*), the divergences among the living groups occurred from more than 300 million to more than 510 million years ago.
12. K. G. Field *et al.*, *Science* **239**, 748 (1988).
13. C. R. Woese, *Microbiol. Rev.* **51**, 221 (1987); R. Cedergren, M. W. Gray, Y. Abel, D. Sankoff, *J. Mol. Evol.* **28**, 98 (1988).
14. The 18*S* rRNA sequences determined range in length from 1769 (*Styela*) to 1959 (*Eptatretus*) nucleotides and consist of all but the extreme 3′ end [39 nucleotides in the human sequence (*15*)]. These sequences have been deposited in the GenBank database under accession numbers M97571 to M97577. All sequences were determined both by direct RNA sequencing with reverse transcriptase and by polymerase chain reaction (PCR) amplification and sequencing of rDNA on the opposite (noncoding) strand. Templates for DNA sequencing were produced by either asymmetric PCR [U. B. Gyllensten and H. A. Erlich, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7652 (1988)] or (in the case of the two hagfishes, where asymmetric PCR did not produce satisfactory results) by cloning PCR products into M13mp19. Ambiguities in DNA sequences were resolved by the use of deoxyinosine triphosphate (dITP). Methods for direct RNA sequencing and asymmetric PCR, as well as primer sequences, are as described (*16*). Amplification of two overlapping segments of hagfish rDNA for cloning was performed with primer pair 20F 5′-GCCGGAGCTCGGTACCTGGTTGATCCTGCCAG-3′ and 429R 5′-GCCGCTGCAGTCGACTTTCTCAGGCTCCCTCTCCGG-3′ and pair 366F 5′-GCCGGAGCTCGGTACCGTCTGCCCTATCAACT-3′ and 1830R 5′-GCCGCTGCAGTCGACACCTACGGAAACCTTGTT-3′, where underlined sequences represent restriction sites added to the primer, numbers indicate the position of the nucleotide at the 3′ end in the human sequence (*15*), and F and R refer to primers that bind to the noncoding and coding strands, respectively. For *Eptatretus*, preliminary sequencing of six clones revealed less than 0.6% sequence difference with the RNA (aside from a single aberrant clone that was 14% different). A consensus for each position was assembled from the RNA sequence and at least two clones. For *Myxine*, two clones of the 20FL-429RL amplification were identical to each other and did not differ from the unambiguous portions of the RNA sequence. Eleven clones of the 366FL-1830RL amplification, however, fell into two sequence classes. Ten of the clones had fewer than 0.7% differences among each other but differed from the RNA sequence by 3.6%, while the remaining clone differed from the RNA by 0.9%. To enrich for clones similar to the RNA, two new primers, 501R 5′-GCCGCTGCAGTTCGTCACTACCTCACCGTG-3′ and 502F 5′-GCCGGGTACCAAATTACCCACTCCCGACA-3′, were designed based on differences between the two classes of clones and used for the amplifications 20F-501R and 502F-1830R. Two clones most similar to the RNA from each amplification (all had <1% difference) were sequenced and, along with the clones from the 20F-429R and the direct RNA sequence, were used to assemble a consensus. The differences among clones in *Myxine* (presumably due to nontranscribed copies of rDNA genes) are not likely to affect phylogenetic analyses because the RNA sequence was ambiguous at 143 out of the 1849 positions reported, and only 8 of these 143 positions were variable among clones.
15. F. S. McCallum and B. E. H. Maden, *Biochem. J.* **232**, 725 (1985).
16. D. W. Stock, K. D. Moberg, L. R. Maxson, G. S. Whitt, *Environ. Biol. Fish.* **32**, 99 (1991).
17. P. M. Ajuh, P. A. Heeney, B. E. H. Maden, *Proc. R. Soc. London Ser. B.* **245**, 65 (1991).
18. J. S. Nelson, *Fishes of the World* (Wiley, New York, 1984).
19. The best outgroup for such comparisons would be the invertebrate group most closely related to vertebrates. The most commonly favored groups are lancelets (*7*) [B. Schaeffer, *Evol. Biol.* **21**, 179 (1987)] or tunicates [R. P. S. Jefferies, *The Ancestry of the Vertebrates* (Cambridge Univ. Press, New York, 1986)], although even arthropods have been suggested (*6*). Views such as the last one that deny the monophyly of the Chordata (vertebrates, tunicates, and lancelets) have not received much support and are contradicted by analyses of 18*S* rRNA (*12*) [C. Patterson, in *The Hierarchy of Life*, B. Fernholm, K. Bremer, H. Jörnvall, Eds. (Elsevier, Amsterdam, 1989), pp. 471–488; J. A. Lake, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 763 (1990); our own unpublished comparisons]. These sequence comparisons either place lancelets closest to vertebrates or place lancelets, tunicates, and vertebrates in an unresolved trichotomy.
20. The gnathostome sequences examined include our own unpublished data.
21. M. D. Hendy and D. Penny, *Syst. Zool.* **38**, 297 (1989).
22. The alignment has been deposited in the ribosomal database project [G. J. Olsen, R. Overbeek, N. Larsen, C. R. Woese, *Nucleic Acids Res.* **19**, 4817 (1991)].
23. D. L. Swofford, *PAUP: Phylogenetic Analysis Using Parsimony Version 3.0r* (Illinois Natural History Survey, Champaign, 1991).
24. J. Felsenstein, *Evolution* **39**, 783 (1985).
25. ———, *PHYLIP Manual Version 3.2* (University Herbarium, University of California, Berkeley, 1989).
26. We thank G. Lecointre, R. Matson, and W. Gobin and the Wisconsin Department of Natural Resources for providing specimens of *Myxine*, *Lampetra*, and *Petromyzon*, respectively, and C. Woese for the gift of several primers. D. Swofford and G. Olsen provided advice on phylogenetic reconstruction, and H. Robertson and D. Swofford read and commented on earlier drafts. This study was supported by NSF grants BSR-87-17417 (to G.S.W.) and BSR-88-15362 (to G.S.W. and D.W.S.) and an NSF predoctoral fellowship (genetics) (to D.W.S.)

30 March 1992; accepted 3 June 1992

Long-Term Survival of Xenogeneic Pancreatic Islet Grafts Induced by CTLA4Ig

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Antigen-specific T cell activation depends on T cell receptor-ligand interaction and costimulatory signals generated when accessory molecules bind to their ligands, such as CD28 to the B7 (also called BB1) molecule. A soluble fusion protein of human CTLA-4 (a protein homologous to CD28) and the immunoglobulin (Ig) G1 Fc region (CTLA4Ig) binds to human and murine B7 with high avidity and blocks T cell activation in vitro. CTLA4Ig therapy blocked human pancreatic islet rejection in mice by directly affecting T cell recognition of $B7^+$ antigen-presenting cells. In addition, CTLA4Ig induced long-term, donor-specific tolerance, which may have applications to human organ transplantation.

At present, the major therapies to prevent the rejection of organ transplants rely on panimmunosuppressive drugs, such as cyclosporine A or monoclonal antibodies (MAbs) to CD3. These drugs must frequently be taken for the life of the individual, depress the immune system, and often result in increased infections and cancer. We attempted to develop a treatment that affected only the transplant antigen-specific T cells, thus inducing donor-specific tolerance. The binding of CD28 by its ligand, B7/BB1 (B7), during T cell receptor engagement is critical for proper T cell signal-

ing in some systems (*1–4*). When the interaction of CD28 with its ligand is blocked, antigen-specific T cells are inappropriately induced into a state of antigen-specific T cell anergy (*1, 5*). Recent studies have shown that the CTLA-4 molecule, a CD28 homolog, also binds to B7 (6). These studies used a soluble chimeric CTLA-4 fusion protein between the variable domain of the human CTLA-4 gene and the hinge, CH2, and CH3 domains of the human IgG1 constant region, CTLA4Ig (6–8). This soluble receptor molecule binds to both human and murine B7 (with a 20-fold greater affinity than CD28), blocks the binding of CD28 to B7, inhibits T cell activation, and induces T cell unresponsiveness in vitro (5, 6, 9). Using a xenogeneic transplant model (*10*), we found that CTLA4Ig prevented rejection of xenogeneic pancreatic islet cells and induced donor-specific tolerance.

Initial studies showed that CTLA4Ig bound to both murine and human B7 and inhibited primary xenogeneic mixed lymphocyte reactions in vitro (*11*). Therefore, we examined the effects of blocking CD28-B7 interaction in vivo. C57BL/6 (B6) or C57BL/10 (B10) mice were treated with streptozotocin to eliminate mouse pancreatic islet β cell function. Diabetic animals were grafted under the kidney capsule, and treatment was started immediately after surgery. Survival of the islet grafts was monitored by the analysis of blood glucose concentrations. Transplanted control animals, treated with either phosphate-buffered saline (PBS) (n = 14) or L6 (a human IgG1 chimeric MAb; n = 8), had a mean graft survival of 5.6 and 6.4 days, respectively (Fig. 1A). In contrast, islet rejection was delayed in animals treated with CTLA4Ig (10 μg per day for 14 days), with four out of the seven animals exhibiting moderately prolonged mean graft survival (12.75 days), whereas the remaining three animals maintained normal glucose levels for >80 days (Fig. 1B). This eventual increase in glucose concentration may be a result of islet exhaustion because no evidence of active cellular rejection was observed. In the three mice that maintained long-term islet grafts, the transient increase in glucose concentrations around day 21 after the transplant may have represented a self-limited rejection episode [consistent with the pharmacokinetics of CTLA4Ig clearance after therapy (*12*)].

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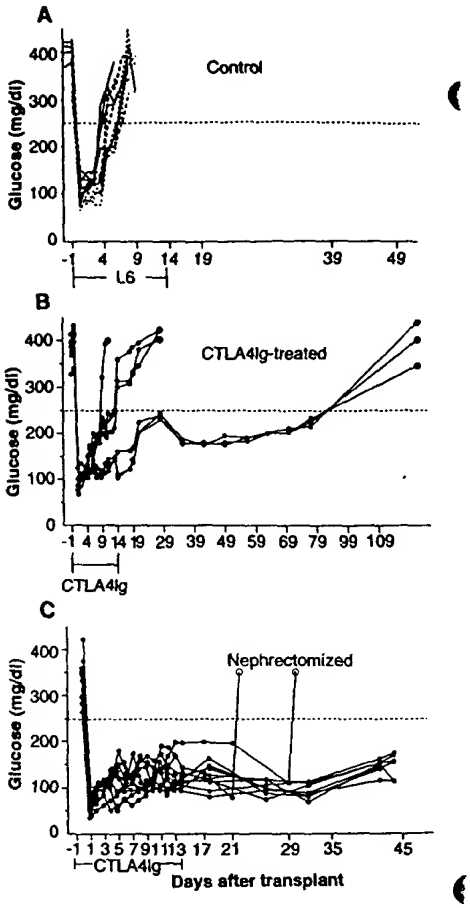

Fig. 1. Survival of human pancreatic islet xenografts. Human pancreatic islets cells were purified after collagenase digestion as described (*17*). B6 or B10 mice, treated with streptozotocin (175 mg per kilogram of body weight) 3 to 5 days before transplant and exhibiting nonfasting plasma glucose levels of greater than 280 mg/dl (with the majority over 300 mg/ml), were used as recipients. Each animal received approximately 800 fresh human islets of 150 μm in diameter beneath the left renal capsule (*10*). Treatment was started immediately after transplantation. (**A**) Control animals were treated with PBS (solid lines) or L6 (dotted lines) at 50 μg every other day for 14 days immediately after transplantation. Islet transplants were considered rejected when glucose levels were greater than 250 mg/dl for three consecutive days. Animals treated with PBS (n = 14) and L6 (n = 8) had mean graft survivals of 5.6 and 6.4 days, respectively. (**B**) Animals were treated with 10 μg of CTLA4Ig for 14 consecutive days immediately after transplant (n = 7). Three out of seven animals maintained their grafts for >80 days. The remaining four animals had a mean graft survival of 12.75 days. (**C**) Animals were treated with 50 μg of CTLA4Ig every other day for 14 days immediately after human islet transplantation. All animals (n = 12) treated with this dose maintained grafts throughout the analysis. Selected mice were nephrectomized on days 21 and 29 after the transplant to assess the graft's function.

Therefore, in subsequent experiments, the dose of CTLA4Ig was increased to 50 μg per animal every other day for 14 days. This treatment resulted in 100% of the animals maintaining normal islet function throughout the experiment with no signs of a rejection crisis (Fig. 1C). In order to confirm that insulin production originated from the transplanted islets and not from the native mouse pancreas, we nephrectomized selected animals at days 21 and 29 to remove the islet grafts (Fig. 1C). In these animals, glucose concentrations increased to above 350 mg/dl within 24 hours, which indicated that the islet xenograft was responsible for maintaining normal glucose levels. Thus, it appears that the blocking of the CD28-B7 interaction inhibits xenogeneic islet graft rejection. The effects of treatment with the soluble receptor were not a result of Fc binding (L6 did not affect graft rejection) or general effects on T cell or B cell function in vivo (*13*). The function of CTLA-4 on T cell surfaces as a potential costimulatory molecule is unknown. Therefore, the effects we observed might also reflect the importance of CTLA-4–B7 interactions or other, as yet unidentified B7 or CTLA-4 ligands.

Histological analyses of islet xenografts from control (PBS-treated) and CTLA4Ig-treated mice were done (Fig. 2). The islet tissue from the control animal demonstrated evidence of immune rejection, with a marked lymphocytic infiltrate into the graft and few remaining islets (Fig. 2A). Immunohistochemical staining showed that insulin-positive cells were present only rarely, and no somatostatin-positive cells (*13*) were present at all (Fig. 2B). In contrast, transplant tissue from the CTLA4Ig-treated mice was devoid of any lymphocytic infiltrate (Fig. 2C). The grafts were intact, with many islets visible. In addition, the β cells observed in the human islet tissue produced human insulin (Fig. 2D) and somatostatin (*13*).

Because the human CTLA4Ig used in this study reacts with both murine and human B7, the individual role of murine $B7^+$ and human $B7^+$ cells could not be distinguished (6). However, one advantage of the xenogeneic transplant model is the availability of a MAb to human B7 that does not react with mouse B7 (*14*). Thus, the role of human B7-bearing antigen-presenting cells (APCs) could be directly examined. The mice were transplanted as described and then treated with 50 μg of MAb to human B7 every other day for 14 days after transplant. This treatment prolonged graft survival in treated mice (9 to

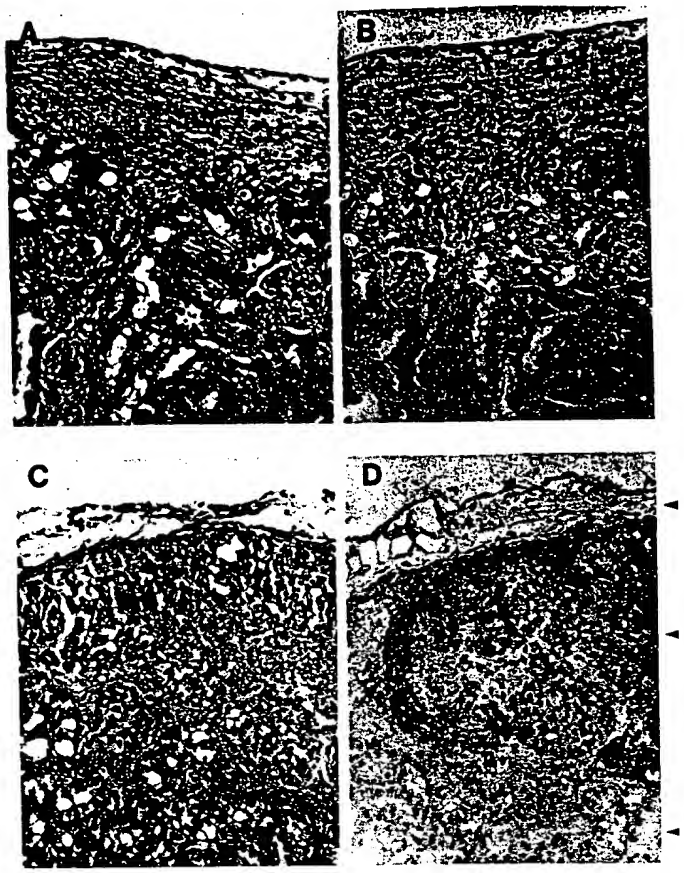

Fig. 2. Histological analysis of human islets transplanted under the kidney capsul of B10 mice. Histology was performed on kidneys transplanted with human islet cells. The slides were analyzed blindly. **(A)** Hematoxylin and eosin staining of a control human islet grafted mouse 29 days after transplantation showed a massive lymphocyte infiltration. **(B)** The same tissue, stained for insulin, showed no detectable insulin production. **(C)** Histological examination of tissue from a CTLA4Ig-treated mouse 21 days after transplant showed intact islets under the kidney capsule with very few lymphocytes infiltrating the transplanted tissue. The tissue was stained with hematoxylin and eosin. **(D)** The same tissue from the CTLA4Ig-treated mouse, stained for insulin, showed the production of insulin by the grafted islets. Similar results were observed in graft tissue examined at latter time points (*13*). The upper, middle, and lower arrowheads identify the kidney capsule, islet transplant, and kidney parenchyma, respectively. All tissues were fixed in 10% buffered formalin and processed, and 5-μm sections were stained either with hematoxylin and eosin or for insulin with the avidin-biotin-peroxidase method (*18*). Magnification is ×122.

>50 days) in comparison to that for control mice (Fig. 3). These results suggest that the immune response to the human islets involves direct presentation of human major histocompatibility complex (MHC)–restricted islet antigens by human APCs. This possibility contrasts with conclusions drawn in previous studies in which the predominant pathway for xenogeneic antigen presentation appeared to involve the processing and presentation of shed foreign proteins by syngeneic mouse APCs (*15*). However, other studies have noted the importance of carrier donor leukocytes in transplant rejection (*16*). The inability of the anti-B7 MAb to block rejection as effectively as CTLA4Ig may indicate that murine $B7^+$ APCs may also be involved in xenograft rejection. It is also possible that an inadequate dose of the anti-B7 MAb was used because it has a lower binding affinity to B7 than to CTLA4Ig (*6*). Further studies are needed to determine how the syngeneic and xenogeneic APCs interact to regulate graft rejection.

Although the CTLA4Ig therapy resulted in graft acceptance in the majority of mice, the animals may not be tolerant. Transient immunosuppression can lead to permanent islet graft acceptance because of graft adaptation (the loss of immunogenicity as a result of the loss of APC function) (*16*). In order to differentiate between these possibilities, we nephrectomized selected xenografted, CTLA4Ig-treated mice (day 40) and retransplanted them under the remaining kidney capsule with either the original donor islets (first party) or unrelated second party human islets (Fig. 4). Streptozotocin-treated control animals, having never received an islet graft, were also transplanted with either first or second party islets. No treatment after the transplant was given. Control animals rejected the first and second party islets by day 4. The CTLA4Ig-treated animals that had received the second party islets rejected these islets by day 5, whereas animals receiving first party donor islets maintained the grafts for >80 days (Fig. 4).

These results suggest that the CTLA4Ig treatment resulted in prolonged donor-specific unresponsiveness to the xenogeneic islets. The ability of the murine immune

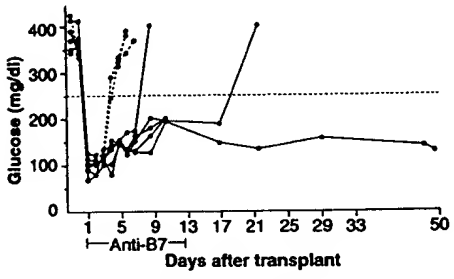

Fig. 3. Prolongation of islet graft survival with MAb to human B7. Streptozotocin-treated animals were transplanted as described (Fig. 1). The mice were treated either with PBS (dotted lines) or with MAb to human B7 (solid lines) at a dose of 50 μg every other day for 14 days. Control animals (treated with PBS) (n = 3) had a mean graft survival of 3.5 days, whereas anti-B7–treated animals (n = 5) maintained grafts from 9 to >50 days.

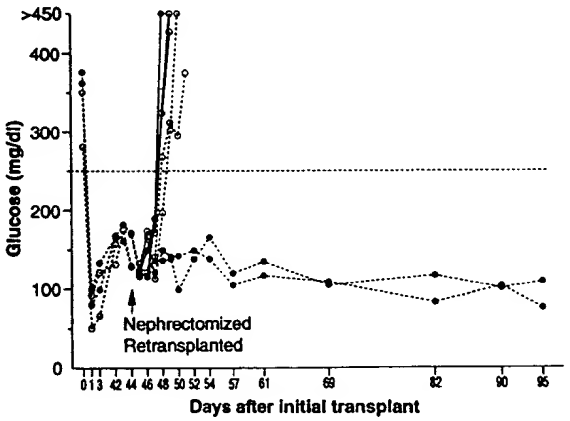

Fig. 4. Induction of donor-specific unresponsiveness to islet graft antigens by CTLA4Ig. Normal glycemic, CTLA4Ig-treated, transplanted mice (dotted lines) were nephrectomized on day 44 after transplant and immediately retransplanted with either 1000 first party donor islets (dotted lines, solid circles) or 1000 second party islets (dotted lines, open circles) beneath the remaining kidney capsule. These islets, frozen at the time of the first transplant, were thawed and cultured for 3 days before transplant to ensure islet function. B10 mice that had been treated with streptozotocin and exhibited nonfasting glucose levels of greater than 280 mg/dl were used as controls (solid lines). No treatment was given after transplantation. Control animals rejected both the first party (solid lines, closed circles) and the second party (solid lines, open circles) islet grafts by day 4 after transplant. The CTLA4Ig-treated mice retransplanted with second party islets had a mean graft survival of 4.5 days, whereas animals retransplanted with first party donor islets maintained grafts for as long as analyzed (>80 days).

response to distinguish differences among the human islet donors also supports the direct recognition of the polymorphic MHC products expressed on the human islet cells. The future of immunosuppressive therapies in transplantation and autoimmune disease depends on their ability to induce long-term, antigen-specific unresponsiveness. The capacity of CTLA4Ig to significantly prolong human islet graft survival in mice in a donor-specific manner suggests that blocking the interaction of costimulatory molecules such as CD28-B7 may provide an approach to immunosuppression.

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Immunosuppression in Vivo by a Soluble Form of the CTLA-4 T Cell Activation Molecule

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In vitro, when the B7 molecule on the surface of antigen-presenting cells binds to the T cell surface molecules CD28 and CTLA-4, a costimulatory signal for T cell activation is generated. CTLA4Ig is a soluble form of the extracellular domain of CTLA-4 and binds B7 with high avidity. CTLA4Ig treatment in vivo suppressed T cell–dependent antibody responses to sheep erythrocytes or keyhole limpet hemocyanin. Large doses of CTLA4Ig suppressed responses to a second immunization. Thus, costimulation by B7 is important for humoral immune responses in vivo, and interference with costimulation may be useful for treatment of antibody-mediated autoimmune disease.

Costimulatory signals delivered by antigen-presenting cells (APCs) have been proposed to control immune responses to transplanted tissues (*1*). Antigenic stimulation of T cells in vitro in the absence of costimulation leads to aborted T cell proliferation and the development of functional unresponsiveness or clonal anergy of T cells (*2*). Several molecules on APCs augment T cell proliferation (*3*, *4*) and regulate functional unresponsiveness in vitro (*4*). The B7 activation molecule binds CD28 (*5*) and delivers a costimulatory signal for T cell proliferation (*6*). T cell–dependent B cell differentiation requires the interaction of B7 with CD28 (*7*). CTLA-4, a T cell molecule homologous to CD28 (*8*), also binds the B7 counter-receptor (*9*). CTLA4Ig, a chimeric immunoglobulin C_γ fusion of CTLA-4, binds with high avidity (dissociation constant ~12 nM) to B7 and potently blocks T cell–dependent immune responses in vitro (*9*). CD28 probably participates in costimulation required to prevent anergy induction in T cell clones (*10*), in unresponsiveness in human mixed lymphocyte reactions (*11*), and in the costimulation of antigen-specific interleukin-2 production of human T cells (*12*). Despite data that indicate the importance of B7-CD28 interactions in the costimulation of in vitro T cell responses, the role of these interactions in regulating in vivo immune responses is unknown. Here, we show that CTLA4Ig is a potent suppressor of antibody responses in vivo.

Human CTLA4Ig [human CTLA-4 and human immunoglobulin (Ig)] binds to murine B7 and inhibits murine T cell responses in vitro (*13*). These findings led us to test the effects of human CTLA4Ig on murine immune responses in vivo. CTLA4Ig was purified to homogeneity by protein A chromatography from a serum-free culture medium of transfected mammalian cells (*14*). The chimeric monoclonal antibody (MAb) L6, which has a murine region and a human Fc region, was used as a control.

We first measured serum clearance of human CTLA4Ig in mice (Fig. 1). A plot of serum CTLA4Ig levels versus time was biphasic, giving a time of half-clearance ($t_{1/2}$) of ~4 hours and ~30 hours for the two components. Serum clearance after multiple injections of CTLA4Ig was more complex and appeared dose-related. The $t_{1/2}$ for the more slowly clearing component was increased to ~4 days after six daily intravenous injections of CTLA4Ig (200 μg per injection), and functionally active CTLA4Ig was detected in mouse serum for up to ~5 weeks after the last treatment with CTLA4Ig. No overt toxicity of CTLA4Ig was noted.

The ability of CTLA4Ig to suppress for-

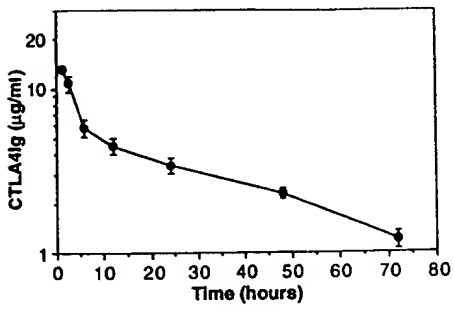

Fig. 1. Serum clearance of human CTLA4Ig in mice. BALB/c mice were each given a single intravenous injection of 50 μg of CTLA4Ig prepared from COS cells. At the indicated times, the mice were bled retro-orbitally. The binding of functional CTLA4Ig from sera to B7$^+$ CHO cells was measured by flow cytometry (*6*). CTLA4Ig concentrations were quantitated by comparison of the degree of binding with the binding of known concentrations of CTLA4Ig. Values represent mean concentrations ± SD from five mice.

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Long-Term Acceptance of Major Histocompatibility Complex Mismatched Cardiac Allografts Induced by CTLA4Ig Plus Donor-specific Transfusion

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Summary

Allograft rejection is a T cell–dependent process. Productive T cell activation by antigen requires antigen engagement of the T cell receptor as well as costimulatory signals delivered through other T cell surface molecules such as CD28. Engagement of CD28 by its natural ligand B7 can be blocked using a soluble recombinant fusion protein, CTLA4Ig. Administration of CTLA4Ig blocks antigen-specific immune responses in vitro and in vivo, and we have shown that treatment of rats with a 7-d course of CTLA4Ig at the time of transplantation leads to prolonged survival of cardiac allografts (median 30 d), although most grafts are eventually rejected. Here, we have explored additional strategies employing CTLA4Ig in order to achieve long-term allograft survival. Our data indicate that donor-specific transfusion (DST) plus CTLA4Ig can provide effective antigen-specific immunosuppression. When DST is administered at the time of transplantation followed by a single dose of CTLA4Ig 2 d later, all animals had long-term graft survival (>60 d). These animals had delayed responses to donor-type skin transplants, compared with normal rejection responses to third-party skin transplants. Furthermore, donor-matched second cardiac allografts were well tolerated with minimal histologic evidence of rejection. These data indicate that peritransplant use of DST followed by subsequent treatment with CTLA4Ig can induce prolonged, often indefinite, cardiac allograft acceptance. These results may be clinically applicable for cadaveric organ and tissue transplantation in humans.

Induction of a T cell immune response is required for allograft rejection (1). Antigen-specific T cell activation is initiated through the TCR (2). However, recent evidence has shown that T cells require two signals for activation (3): signal 1, which is provided by stimulation through the TCR, and signal 2 (costimulation), which can be provided by ligation of one or more T cell surface receptors. Engagement of the TCR receptor alone (i.e., signal 1 without signal 2) has been reported to induce a long-lasting state of T cell anergy, rendering cells unresponsive to subsequent antigenic stimulation (3).

The best characterized costimulatory pathway is transduced through the CD28 surface molecule. CD28 is a receptor for B7, a molecule that can be expressed by many types of APCs (4). Stimulation of CD28 by its ligand B7 provides sufficient costimulatory signals to TCR-activated T cells for lymphokine production and cell proliferation (5). CTLA-4 is a gene closely related to CD28 which also serves as a B7-ligand, although the physiologic role of this interaction is not yet defined (6). Recently, a soluble recombinant protein termed CTLA4Ig was produced, which contains the extracellular domain of human CTLA-4 fused to a human Ig Cγ chain (6). CTLA4Ig displays an ~20-fold higher affinity for B7 than does CD28 (6), therefore acting as a competitive inhibitor of CD28 engagement. Although constructed using the human CTLA-4 domain, CTLA4Ig binds efficiently to murine and rat B7. CTLA4Ig inhibits B7-dependent immune responses in vitro (6). In vivo, CTLA4Ig blocks T cell–dependent B cell antibody production, and prevents the rejection of xenogeneic islet and allogeneic cardiac allografts (7–9). In the studies of cardiac allograft rejection we found that animals treated with daily injections of CTLA4Ig for 7 d, initiated at the time of transplantation, had greatly prolonged graft survival (median 30 d, versus 7 d for control animals), although most animals eventually rejected the graft (7). Pretransplant thymectomy did not further extend survival in CTLA4Ig-treated

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animals, indicating that T cells present in the animals at the time of transplantation eventually recovered the capacity to induce rejection.

In this report we have explored other strategies using CTLA4Ig in order to achieve consistent long-term allograft survival. We find that donor-specific cell transfusion (DST) at the time of transplantation, followed by a single dose of CTLA4Ig 2 d later, is sufficient to lead to prolonged, often indefinite, cardiac allograft survival. Since no treatment is required before transplantation, these results may be clinically applicable for cadaveric organ and tissue transplantation in humans.

Materials and Methods

Animals. The experiments were conducted using inbred male Lewis (LEW, RT1^l), Brown Norway (BN, RT1^n) and ACI (RT1^a) rats weighing 200–300 g (Harlan Sprague Dawley, Inc., Indianapolis, IN). LEW rats served as heterotopic cardiac allograft recipients. BN and ACI rats were used as cardiac and skin allograft donors, and as a source of splenocytes for DST.

Splenocyte Transfusion. Spleens were harvested and a single cell suspension prepared as previously described (7). Red cells were lysed with water, and the remaining population of mononuclear cells was washed two times in RPMI-1640. Cell viability was consistently >95% as determined by trypan blue exclusion. Varying numbers of splenocytes were injected intravenously in a volume of 0.5–1.0 ml into anesthetized rats at the indicated time points.

Cardiac Transplantation. The rats were anesthetized and mechanically ventilated, after which donor hearts were transplanted into a cervical location in the recipient animals (10). Allograft survival was assessed by daily palpation. The day of rejection was defined as the day of cessation of palpable heartbeat, and verified by autopsy and selective pathological examination. Loss of graft function within 48 h of transplant was considered a technical failure (<5% on the average), and these animals were omitted from further analysis.

Skin Grafting. Animals with long-term (>60 d) cardiac allograft survival received donor-specific and third-party skin grafts placed simultaneously on alternate sides of the flanks. Donor skin was raised as full-thickness grafts from the center abdominal wall, trimmed of fat, cut into standard sizes (2 cm circular skin), and sutured into position as described (11). The grafts were inspected daily, and rejection was said to have occurred when more than 50% of the graft surface became raised, necrotic, or covered by eschar.

Mixed Lymphocyte Reaction (MLR). Lymphocytes were isolated from cervical and axillary nodes by gentle passage of tissue through a nylon mesh. Cells were cultured in RPMI-1640 medium supplemented with 5 mM Hepes, penicillin (10^5 U/liter), streptomycin (100 μg/liter), 50 μM 2-ME, and 10% FCS (GIBCO BRL, Gaithersburg, MD). Next, 3×10^5 each of responder cells and irradiated (3,000 rad; ^{137}Cs source) stimulator cells were cocultured for 4 d in 96-well flat-bottomed microtiter plates as described (7). Proliferation, measured as DNA synthesis, was determined by adding 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) to each well for the last 6 h of culture. All assays were performed in quadruplicate.

Results and Discussion

In our initial studies, we found that a 7-d course of CTLA4Ig administered to cardiac allograft recipients starting at the time of transplantation significantly prolonged allograft survival but did not prevent eventual rejection (7). As prior thymectomy failed to induce permanent engraftment in treated animals, T cells present during the course of CTLA4Ig administration were responsible for rejection. We hypothesized that the kinetics of T cell trafficking might not permit all alloreactive cells to encounter donor antigens in the graft or regional lymphoid tissue during the 7-d period of drug administration, and that T cells that escaped an encounter with antigen in the context of B7-blockade could eventually reject the graft. Therefore, we first extended the period of CTLA4Ig treatment. However, animals receiving daily CTLA4Ig for 21 d rejected their grafts at the same time points as those treated for only 7 d (data not shown).

We next considered the possibility that immunosuppression would be most effective if the alloantigen were administered systemically in conjunction with CTLA4Ig, so as to expose all T cells throughout the body to antigen during the drug treatment period. For example, treatment of mice with CTLA4Ig blocks the immune response to systemically administered antigens such as sheep RBCs or KLH (8). Therefore, LEW animals received 10^8 BN lymphocytes intravenously (DST) plus a single dose of CTLA4Ig (0.5 mg) 14 d before placement of a BN cardiac allograft. As shown in Table 1, animals treated with this protocol had significant prolongation of cardiac allograft survival, with median graft survival of 17 d. This effect required both DST and CTLA4Ig, as animals treated with either alone rejected their grafts by 8 d. Furthermore, immunosuppression by DST plus CTLA4Ig was antigen specific. Animals receiving transfusions from third-party ACI animals had a median graft survival of only 9 days. Conversely, when ACI animals were used as heart donors, DST from ACI animals synergized with CTLA4Ig to prolong graft survival (≥20 d), whereas transfusions from BN animals had no effect (rejection by day 7). These data also indicate that CTLA4Ig is not merely acting by depleting the graft of B7$^+$ APCs. First, strain-specific cell transfusions are required for the immunosuppressive effect. Second, our prior analysis of CTLA4Ig pharmacokinetics indicated a serum half-life of 2.8 d (7), and therefore by the time of transplantation circulating CTLA4Ig levels would have fallen far below a therapeutic level.

We had initially chosen to administer CLTA4Ig plus DST 14 d before transplantation because of the possibility that it might take several days after exposure to this regimen for a state of antigen-specific nonresponsiveness to develop. However, although graft survival was significantly prolonged in animals treated with this protocol, eventually most grafts were rejected. Therefore, we next considered whether a state of tolerance was being induced, but that this state was temporary, and had largely waned by 2 wk. This concept was supported by two sets of data. First, in studies of anergy induction in T cell clones, anergy, although reproducible, was not a permanent state, as the cells could spontaneously regain responsiveness after a finite period of time (12). Second, recent in vivo studies have demonstrated that maintenance of anergy requires the persistence of antigen (13). Thus we reasoned that induction of anergy by DST plus CTLA4Ig might best

Table 1. *Effects of Pretransplant DST plus CTLA4Ig on Cardiac Allograft Survival*

Heart donor	DST donor	DST day	CTLA4Ig day	Graft survival
				d
BN	None			5,7,7,7,7,7,7,7,7,7
BN	BN	−14		6,6,7,7,8,8
BN	None		−14	6,8,8
BN	BN	−14	−14	14,14,15,16,17,19,46,>100
BN	ACI	−14	−14	7,8,9,9,10,13
ACI	None			5,5,6,7,8
ACI	ACI	−14	−14	23,26,58,>100
ACI	ACI	−14	None	1,2,5,5,6,7
ACI	BN	−14	−14	6,6,7,7,7

Allograft recipients were LEW animals, some of which received DST (10^8 cells) via intravenous injection, CTLA4Ig (0.5 mg) by intraperitoneal injection, or both 14 d before transplantation as indicated. The day of transplantation was defined as day 0.

prolong graft survival if transplantation was performed simultaneously. However, when CTLA4Ig plus DST (4×10^7 cells) were administered on the day of transplantation, graft survival was not improved over the previous protocol, and was no different than in animals treated with a single dose of CTLA4Ig without DST (Table 2).

At present, knowledge of the target of action of CTLA4Ig is limited. Its only known ligand is B7, whose expression is restricted to cells of hematopoietic origin, in particular those which can serve as APCs. In the case of vascularized organ allografts, there are two routes of alloantigen presentation using distinct APCs (14, 15). In the indirect route, donor alloantigens, shed from the surface of donor parenchymal cells, are presented by host APCs in regional LNs. In the direct route, APCs resident within the graft directly stimulate host T cells. Although the relative contribution of these two routes is the subject of debate, the importance of direct sensitization has been demonstrated by the fact that graft survival is significantly prolonged by depleting tissues and organs of resident APCs (for a review see reference 16). In this regard, it is notable that whereas a short course of CTLA4Ig frequently induced indefinite survival of human islet xenografts in mice, anti–human B7 antibody was effective as well, indicating that much of the immune response was initiated against donor-type APCs (9).

One of the most potent types of APC known is the dendritic cell (17). Compared with activated B cells and macrophages, extremely small numbers of dendritic cells are sufficient to stimulate T cell responses. Furthermore, in contrast to B cells and macrophages, dendritic cells constitutively express B7 (18, 19). Larsen et al., (20) have shown that after cardiac allograft placement, donor dendritic cells migrate into host lymphoid organs, and it is possible that this may be responsible for initiating graft rejection. The migration of dendritic cells to LNs was found to peak 2 d after transplantation. Therefore, we next administered DST (4×10^7 cells) on the day of transplantation, followed by a single dose of CTLA4Ig 2 d later (Table 2). Whereas DST alone was not immuno-

Table 2. *Effects of Peri-transplant DST plus CTLA4Ig on Cardiac Allograft Survival*

DST donor	DST day	CTLA4Ig day	Graft survival
			d
–	–	–	5,7,7,7,7,7,7,7,7,7
BN	0	–	7,7,9,10,11
–	–	0	8,10,18
BN	0	0	8,11,19
–	–	2	8,14,17,25,28
BN	0	2	89,>100,>100,>100,>100
ACI	0	2	14,23,26,28,56

Allograft recipients were LEW animals and heart donors were BN animals. Selected recipient animals received DST (4×10^7 cells) via intravenous injection, CTLA4Ig (0.5 mg) by intraperitoneal injection, or both at the times indicated. The day of transplantation was defined as day 0.

Tabl 3. *Skin Graft Survival in Rats with Long-Term Functioning Cardiac Allografts*

Animal	Day of skin grafting	Skin graft survival	
		BN skin	Third-party (ACI) skin
1	64	19	10
2	64	19	10
3	66	22	9
4	72	19	
5	83	19	

Skin graft recipients were LEW animals that had received BN cardiac allografts and immunosuppression with DST plus CTLA4Ig. Skin grafts from donor-strain animals (BN) and from third-party animals were performed simultaneously on the indicated day after cardiac transplantation.

suppressive, and CTLA4Ig by itself had a modest effect on graft survival, the combination reproducibly induced long-term engraftment. Similar to pretransplantation DST plus CTLA4Ig (Table 1), the immunosuppressive effect of this protocol was also antigen specific, as LEW rats that received transfusions from ACI animals rejected BN cardiac allografts at a median of 26 d.

Additional transplants were performed on these animals with stable allografts. Skin transplants from donor-type BN animals or from third-party ACI animals were simultaneously placed on opposite flanks (Table 3). The ACI grafts were all rejected by 10 d, the normal time for rejection of ACI skin grafts in LEW animals. In contrast, the BN grafts survival for a median of 19 d. This demonstrates that these animals remained immunocompetent, yet had donor-specific hyporesponsiveness. In four of five rats, rejection of the BN skin grafts did not induce rejection of the BN hearts, although it is notable that the single animal that rejected its primary

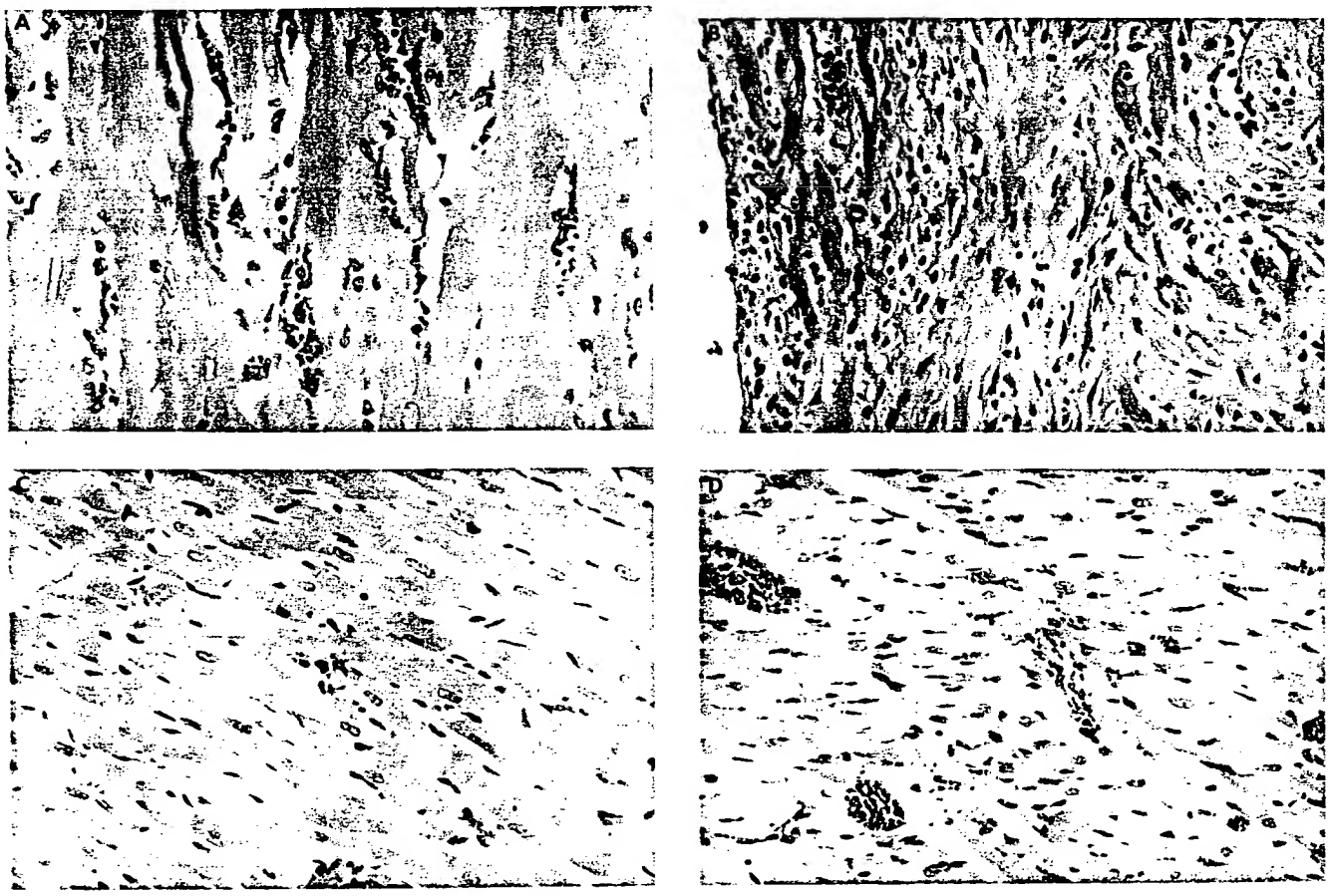

Figure 1. Histopathology of cardiac tissue. Animals were treated with DST on day 0 plus CTLA4Ig on day 2, and hearts were removed for examination at least 60 d after transplantation. First cardiac allografts appeared either histologically normal (*A*) or showed evidence of focal rejection with resulting myocyte loss (*B*). In all cases, second cardiac allografts showed no or minimal histologic evidence of rejection (*C*). The native heart from a LEW animal which received a BN allograft is shown for comparison (*D*). The hearts were fixed in formalin and tissue sections were stained with hematoxylin and eosin. ×100.

cardiac allograft (on day 89) did so 6 d after rejection of a BN skin graft.

Skin grafts are frequently rejected in animals tolerant to vascularized organs, perhaps because of the increased immunogenicity of skin or the existence of skin-specific antigens (21). Second cardiac allografts from BN donors placed into CTLA4Ig plus DST-treated animals, all appeared to function normally until the animals were killed for humane reasons. This occurred on days 17–52 after transplantation of the second heart. In all instances, histologic examination of the second heart revealed only minimal cellular infiltrates consistent with normal histology or focal mild rejection (Fig. 1). Consistent with this minimal response, lymphocytes from engrafted animals have a 50% reduction in their in vitro proliferative response (as measured in an MLR) to donor-type stimulators (data not shown). This data is in agreement with our previous study which found a 50% decrease in MLR responsiveness to donor-type cells in CTLA4Ig-treated animals, with no effect on third-party responses (7). The reduction in proliferation appears to be somewhat modest, given the failure of the animals to reject their cardiac allografts grafts. This suggests either a discrepancy between in vitro and in vivo antidonor reactivity, or split tolerance characterized by CTL activity that is severely depressed in comparison with proliferative capacity.

Together, our data indicate that DST at the time of cardiac transplantation followed by treatment with CTLA4Ig are sufficient to induce long-term cardiac allograft acceptance in rats. These animals remain immunocompetent, but exhibit donor-specific nonresponsiveness. It should be emphasized that although the hearts remain functional as assessed by electrical activity and palpable contractions, some of the grafts have experienced focal rejection with resulting myocyte loss (Fig. 1 *B*). Thus long-term engraftment is not strictly equated with immunologic nonresponsiveness. However, even in animals with significant myocardial damage to their initial allograft, the second graft had minimal histologically abnormalities, suggesting that a previously existing immune response might be relatively quiescent.

A curious finding was that CTLA4Ig was dramatically more effective when given on day 2, than on day 0. Given that the half-life of CTLA4Ig is >60 h, this finding cannot be due to the fact that when administered on day 0, the drug is absent by day 2. Rather, it suggests that the drug is most effective when given after an initial immune response is allowed to begin. The finding that a single dose of CTLA4Ig, without any DST, is more effective when administered at day 2 than at day 0 is consistent with this hypothesis. DST at day 0, which is also required for optimal graft prolongation, may serve to help synchronize and/or maximally stimulate the immune response by distributing alloantigen throughout the lymphoid system. Subsequent treatment with CTLA4Ig could then block costimulatory signals at at time when T cells were maximally sensitive to this maneuver.

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Appendix D

In Vivo Blockade of CD28/CTLA4: B7/BB1 Interaction With CTLA4-Ig Reduces Lethal Murine Graft-Versus-Host Disease Across the Major Histocompatibility Complex Barrier in Mice

By Bruce R. Blazar, Patricia A. Taylor, Peter S. Linsley, and Daniel A. Vallera

We tested whether the in vivo infusion of recombinant, soluble CTLA4 fused with Ig heavy chains, as a surrogate ligand used to block CD28/CTLA4 T-cell costimulation, could prevent efficient T-cell activation and thereby reduce graft-versus-host disease (GVHD). Lethally irradiated B10.BR recipients of major histocompatibility complex disparate C57BL/6 donor grafts received intraperitoneal injections of human CTLA4-Ig (hCTLA4-Ig) or murine CTLA4-Ig (mCTLA4-Ig) in various doses and schedules beginning on day −1 or day 0 of bone marrow transplantation (BMT). In all five experiments, recipients of CTLA4-Ig had a significantly higher actuarial survival rate compared to mice injected with an irrelevant antibody control (L6) or saline alone. Survival rates in recipients of hL6 or PBS were 0% at 29 to 45 days post-BMT. In recipients of CTLA4-Ig, survival rates were as high as 63% mice surviving 3 months post-BMT. However, protection was somewhat variable and recipients of CTLA4-Ig were not GVHD-free by body weight, clinical appearance, and histopathologic examination. There were no significant differences in the survival rates in comparing injection dose, injection duration, or species of CTLA4-Ig (hCTLA4-Ig *v* mCTLA4-Ig). Splenic and peripheral blood flow cytometry studies of long-term hCTLA4-Ig–injected survivors showed a significant peripheral B-cell and CD4$^+$ T-cell lymphopenia, consistent with GVHD. A kinetic study of splenic reconstitution was performed in mice that received hCTLA4-Ig and showed that mature splenic localized CD8$^+$ T-cell repopulation was not significantly different in recipients of hCTLA4-Ig compared with hL6, despite the significant increase in actuarial survival rate in that experiment. These data suggest that the beneficial effect of hCTLA4-Ig on survival is not mediated by interfering with mature donor-derived T-cell repopulation post-BMT. Neither hCTLA4-Ig nor mCTLA4-Ig interfered with hematopoietic recovery post-BMT. We conclude that CTLA4-Ig (most likely in combination with other agents) may represent an important new modality for GVHD prevention.
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LETHAL GRAFT-VERSUS-HOST disease (GVHD) occurs in a high proportion of humans who receive matched sibling or unrelated donor transplants despite conventional chemotherapeutic agents.[1-3] Although in vitro T-cell depletion can eliminate GVHD in recipients of histocompatible sibling donor grafts and significantly reduce severe GVHD in recipients of unrelated donor transplants,[4] post-bone marrow transplantation (BMT) complications including failure to engraft, relapse, and infectious deaths are apparently increased.[2] Additional GVHD preventive strategies are clearly needed.

Recently, our knowledge about T-cell signaling pathways has been substantially extended by the discovery that efficient T-cell activation requires triggering of the antigen-specific T-cell receptor (TCR) and costimulation. T-cell costimulation can be provided either by the binding of the structurally related CTLA4 and CD28 receptors on T cells[5-24] to one of at least two known CD28/CTLA4 counter-receptors on murine antigen-presenting cells (APCs)[25] or by other non-B7 molecules present on APCs.[26,27]

Jenkins, Schwartz, and colleagues[28-32] have shown that interference with costimulation of T-cell activation in antigen-driven murine T-cell clones can render these cells incapable of recognizing antigen even though the clones retain proliferative capacity.[28-32] This state has been termed anergy. Induction of anergy in GVHD-causing cells could be used to prevent donor T cells from responding to antigens/peptides present in GVHD target tissues. Because nonresponsiveness could theoretically be conferred exclusively to GVHD antigens/peptides, other properties of donor T cells such as antiviral and perhaps antitumor immune responsiveness to later challenges in vivo could be retained.

Anergy induction can be accomplished by preventing APCs from providing the necessary costimulatory molecules to T cells or by directly blocking the binding of CD28 and CTLA4 to B7/BB1 using nonactivating monoclonal antibodies (MoAbs) or F(ab')fragments to determinants on the T cell or by exposing T cells to APCs that do not have sufficient amounts of available B7/BB1 to provide costimulation.[13-32] Blockade of B7/BB1 binding to CD28 and CTLA4 has been shown to interfere with costimulatory function.[18] Such blockade has been accomplished by binding of a surrogate CTLA4 ligand to B7/BB1, which has been shown to block T-dependent in vitro immune responses.[18,33,34] A fusion protein consisting of the extracellular domain of CTLA4 linked to the hinge region of Ig heavy chain, referred to as CTLA4-Ig, would facilitate the in vivo inhibition of the CD28:CTLA4 interaction with B7/BB1 by prolonging the half-life of recombinant CTLA4. In vivo infusion of CTLA4-Ig has been shown to reduce antibody responses to sheep red blood cells (RBCs) in mice,[35] permit acceptance of human pancreatic islet cell xenografts in mice,[36] and reduce[37] or eliminate[38]

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rejection of rat cardiac allografts. In the xenogeneic setting, rechallenge experiments indicated that donor-specific xenograft acceptance could be achieved.

Therefore, we undertook studies to test the efficacy of CTLA4-Ig fusion protein as a means of preventing lethal GVHD in murine recipients of fully allogeneic donor grafts. This report will detail our experience with human CTLA4-Ig (hCTLA4-Ig) and murine CTLA4-Ig (mCTLA4-Ig) fusion proteins administered systemically beginning on or immediately before the day of BMT.

MATERIALS AND METHODS

Mice. B10.BR/SgSnJ (H-2^k) recipient mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 (H-2^b) or C57BL/6-Ly5.2 (H-2^b) donor mice were purchased from the National Institutes of Health (Bethesda, MD). C57BL/6 donors and all recipients were female and C57BL/6-Ly5.2 mice were males. Donors were 4 to 6 weeks old and recipients were 8 to 10 weeks old at the time of BMT.

BMT. Our transplant protocol has been described in detail.[39] B10.BR recipients were conditioned with 8.0 Gy (unless otherwise noted) total body irradiation (TBI) administered from a Philips RT 250 Orthovoltage Therapy Unit (Philips Medical Systems, Brookfield, WI) filtered through 0.35 mm Cu at a final absorbed dose rate of 0.41 Gy/min at 225 kV and 17 mA. Eight-Gray TBI was chosen for conditioning based on previous studies which showed that this dose was sufficient for generation of GVHD, not detrimental to survival if irradiated recipients were rescued with anti-Thy1.2 + C′–treated BM, and uniformly lethal in studies in which 8.0 Gy TBI was administered to nonconcurrent control B10.BR mice that were not rescued with BM.[40,41] Briefly, donor BM was collected into RPMI 1640 medium by flushing it from the shafts of femurs and tibias. The cells were resuspended.

Recipients (five to eight mice per group per experiment) received 25×10^6 BM cells from C57BL/6 donors that had been T-cell depleted (TCD) with anti-Thy1.2 (antibody 30-H-12, rat IgG2b; provided by Dr David Sachs, Cambridge, MA) + C′ as previously described.[39-41] Single-cell suspensions of splenocytes were obtained (as a source of GVHD-causing T cells) from C57BL/6 or C57BL/6-Ly5.2^+ donors by passing minced spleens through a wire mesh and collecting them into RPMI 1640. Clumps of debris were allowed to settle out. Splenocytes were suspended with BM at a concentration of 10^8 cells/mL. In congeneic donor transfer experiments, recipients received BM cells from C57BL/6 (Ly 5.1^+) donors and splenocytes from C57BL/6-Ly 5.2^+ donors. All mice received 25×10^6 BM cells and 25×10^6 splenocytes (BMS) via caudal vein injection in 0.5 mL vol.

Ig fusion proteins: Generation, purification, and administration post-BMT. Human CTLA4Ig was produced by stably transfected Chinese hamster ovary (CHO) cells as previously described.[33,36] Human L6 or mouse L6 Ig control was provided by Drs I. and K.I. Hellstrom (Bristol-Meyers-Squibb). A cDNA construct encoding murine CTLA4Ig was assembled in similar fashion as described for human CTLA4Ig from DNA encoding the extracellular region of murine CTLA-4.[10] Concentrations of murine CTLA4Ig were determined by an extinction coefficient at 280 nm of 1.6 (experimentally determined by amino acid analysis of a solution of known absorbance). Binding activity to B7 molecules was essentially identical for all lots of CTLA4-Ig. Endotoxin concentrations were less than 1 endotoxin unit/mg of protein for all CTLA4-Ig lots.

CTLA4-Ig administration. For in vivo administration, mice were injected intraperitoneally (IP) with hCTLA4-Ig, mCTLA4-Ig, the species-appropriate irrelevant MoAb control (hL6 or mL6), or phosphate-buffered saline (PBS) beginning 1 day before or on the day of BMT. Doses were either 100 or 250 μg/injection administered every other day or daily during the first 15 days post-BMT and then daily, every other day, or thrice weekly for up to 2 additional weeks as indicated. To determine CTLA4-Ig levels, sera were obtained by retro-orbital venipuncture and reacted against B7-transfected CHO cells as previously described.[35] Mean channel fluorescence was determined and converted into microgram-per-milliliter concentration values using known concentrations of purified hCTLA4-Ig as a standard.

As a positive control (for GVHD prevention) in all experiments, one experimental group received spleen in which T cells were eliminated with anti-Thy1.2 + C′. We have routinely observed that anti-Thy1.2 + C′ eliminated all detectable natural killer (NK) cell function[42] and at least 95% of cytolytic T-lymphocyte precursors (CTLp).[39]

Immunofluorescence. MoAbs were directly labeled with biotin, fluorescein isothiocyanate (FITC), or phycoerythrin (PE) as previously described.[41] For the biotin-labeled MoAb, fluorescence was indirectly measured by adding streptavidin-labeled Red 613 (GIBCO Laboratories, Grand Island, NY). For chimerism studies, peripheral blood mononuclear cells were isolated and costained with anti–H-2^b-SA-PE (clone EH144, mouse IgG; provided by Dr T.V. Rajan, Albert Einstein University, New York, NY) and anti–H-2^k-FITC (clone 11-4.1, mouse IgG2a; American Tissue Type Culture Collection, Rockville, MD). For thymocyte experiments, two-color flow cytometry studies were performed. For sequential splenocyte and BM experiments, two-color or three-color flow cytometry studies were performed. For flow cytometry studies, single-cell suspensions of splenocytes or BM cells were washed and suspended in buffer (PBS + 5% colostrum-free bovine serum + 0.015% sodium azide). Pelleted cells were incubated for 15 minutes at 4°C with 0.4 μg of an anti-Fc receptor MoAb (clone 2.4G2; provided by Dr Jay Unkless, Rockfeller University, New York, NY) to prevent Fc binding.[43] Optimal concentrations of biotin-, PE-, and FITC-labeled MoAb were added to a total volume of 100 to 130 μL and incubated 1 hour at 4°C. Cells were washed and SA-Red 613 was added for an additional 1 hour at 4°C. After final washing, cells were fixed in 1% paraformaldehyde.

The following antibodies and reagents were used for these studies: Anti-Ly 5.2 (clone A20-1.7, rat IgG2a), anti-Ly 5.1 (clone 104-2, rat IgG2a), both provided by Dr U. Hammerling (New York, NY) for experiments involving congeneic transfer of allogeneic BMS; anti-CD8 (clone 53-6.72, rat IgG2a; provided by Dr Jeffrey Ledbetter, Bristol-Myers-Squibb, Seattle, WA)[44]; anti-CD4 (GK1.5; provided by Dr Frank Fitch, University of Chicago, Chicago, IL)[45]; an irrelevant rat IgG2 antihuman antibody (3A1E)[46] was used as a negative control in all experiments. In some experiments, additional tubes included the following PharMingen (San Diego, CA) MoAbs: anti-TCR α/β (clone H57-597, hamster IgG),[47] anti-CD3ϵ (clone 145-2C11, hamster IgG),[48] B220 (clone Ra3-6B2, rat IgG2a),[49] CD45R (clone 16A, rat IgG2a),[50] CD28 (clone 37.51, hamster IgG),[16,17] interleukin-2R (IL-2R) (clone 7D4, rat IgM),[51] TCR γ/δ (clone GL3, hamster IgG),[52] NK1.1 (clone PK136, mouse IgG2a),[53] CD69 (clone H1.2F3, hamster IgG),[54] and Mac1 α subunit (CD18, clone M1/70.15.11, rat IgG2b).[55]

Hematologic evaluation of recipients post-BMT. Fifty microliters of peripheral blood was obtained by retro-orbital venipuncture on days 28 and 90 post-BMT. Leukocyte number and morphology were determined by examination of Wright-Giemsa–stained slides.[39] Hematocrit percentages were determined by capillary tube RBC-to-plasma volume ratios after centrifugation.

Pathologic examination of tissues. Mice were killed, autopsied, and tissues were taken for histopathologic analysis. All samples were placed in 10% neutral buffered formalin, imbedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopatho-

logic assessment. Organs were scored positive for GVHD if there was single-cell necrosis (skin, colon), crypt dropout (colon), periportal infiltrate with acute necrosis (liver), or endothelialitis with a lymphocytic infiltrate (lung).[56,57] In previous studies, these features were present only in mice with active GVHD and not in normal mice or in irradiated recipients of syngeneic BMT.

Statistical analyses. Group comparison of continuous data were made by Student's *t*-test. Survival data were analyzed by lifetable methods using the Mantel-Peto-Cox summary of χ^2.[58] Probability (P) values $< .05$ were considered significant.

RESULTS

hCTLA4-Ig is partially effective in preventing lethal GVHD induced across the full major histocompatibility complex (MHC). For our initial experiments, we chose to infuse hCTLA4-Ig at 100 μg every other day through day 25 post-BMT based on achievable sera concentrations[35] and known in vivo half-life determinations (approximately 2.8 days).[34] Six or eight mice per group received transplants. Recipients of hCTLA4-Ig had a significant ($P = .0033$) increase in 96-day actuarial survival rate compared with recipients of the irrelevant, species-specific isotype control MoAb (hL6) (67% v 0%, respectively) (Fig 1A; Table 1, experiment 1). hCTLA4-Ig–treated mice survived at a lower rate than recipients of anti-Thy 1.2 + C′-treated cells (100% actuarial survival rate, $P = .074$, compared with hCTLA4-Ig) and had weight loss (Fig 1B) and a clinical appearance consistent with GVHD.

On day 90 post-BMT, four hCTLA4-Ig–treated mice (mean weight = 20.0 g) and six recipients of anti-Thy 1.2 + C′-treated (TCD) BMS (mean weight = 27.2 g) were killed for histopathologic analysis of GVHD target organs. Histologic examination documented that GVHD was present in all recipients of hCTLA4-Ig, involving the liver, lung, and colon, but was absent in recipients of TCD BMS.

Flow cytometry analysis of day 96 post-BMT was performed on spleen, thymus, peripheral blood, and BM from individual mice in these two groups (from Fig 1). Compared with recipients of in vitro TCD BMS, there was a trend toward lower splenocyte numbers in recipients of hCTLA4-Ig and significant reductions in the absolute numbers of B lymphocytes and $CD4^+$ cells (Table 2), involving both $CD45R^+$ (memory cells) and $CD45R^-$ cells to similar degrees (data not shown). No significant differences were noted in the number of splenic $CD8^+$ cells, TCR α/β^+ $CD4^-CD8^-$ (cells with putative counterregulatory properties),[59,60] or $Mac1^+$ (myelomonocytic) cells between these two groups (Table 2). The number of splenic $CD3\epsilon^+NK1.1^+$,[61] $CD25^+$ (IL-$2R^+$) T cells, $CD69^+$ T cells, and TCR γ/δ^+ cells was also not significantly different between the two groups, whereas $CD3\epsilon^-$ $NK1.1^+$ cells were modestly higher in the TCD BMS group (data not shown). Host cells (averaging 1% of the total population) were detectable only in recipients of anti-Thy1.2 + C′–treated BMS.

Peripheral blood mononuclear cells analysis was similar to the splenic analysis demonstrating significant reductions in the proportions of B lymphocytes and $CD4^+$ T cells but not $CD8^+$ T cells. Thymic flow cytometry analysis did not show any reductions in the absolute number of mature $CD3\epsilon^+CD4^+CD8^-$ T cells. BM flow cytometry studies also did not show any significant differences in the number of B or T lymphocytes, TCR$\alpha/\beta^+CD4^-CD8^-$, cells or Mac1+ cells between these two groups. Thus, there was a peripheral but not central reduction in the number of mature B lymphocytes and $CD4^+$ T cells.

A higher hCTLA4-Ig dose does not improve GVHD-free survival. In experiment 1 (Table 1; Fig 1) described above, CTLA4-Ig levels were measured post-BMT during (day 21 post-BMT) and 7 days postinfusion termination. On day 21 post-BMT, five of six mice initially analyzed had levels of 15 μg/mL within the range of previous reports in normal mice,[35] whereas the remaining mouse had a sera level of 4.5 μg/mL hCTLA4-Ig. In vitro, hCTLA4-Ig at concentrations of 1 to 5 μg/mL completely suppresses the mixed lymphocyte response of donor strain C57BL/6 splenocytes toward irradiated host strain B10.BR splenocytes (data not shown). On day 35 post-BMT (7 days after discontinuation of hCTLA4-Ig injections), three of these six mice had nondetectable (<0.1 μg/mL) sera hCTLA4-Ig levels and the remaining three had 0.25, 1.25, or 5 μg/mL. Two of the three mice with the highest levels on day 28 survived the observation period, whereas two of three mice with the lowest levels died 2 to 4 weeks later. These data suggested that mice might benefit from a higher dose of hCTLA4-Ig that permit a more prolonged period of sustained hCTLA4-Ig levels.

Therefore, we increased the dose of hCTLA4-Ig to 250 μg/injection and began injections 1 day pre-BMT so that mice would have circulating hCTLA4-Ig on the day of BMT and continued every other day through day 25 post-BMT. Seven or eight mice per group received transplants (Table 1, experiment 2). Actuarial survival rate was not further improved with 250 μg per injection compared with 100 μg per injection in the first experiment. Although the actuarial survival rate was significantly ($P = .042$) higher in recipients of hCTLA4-Ig compared with hL6, only 13% of hCTLA4-Ig survived the 2-month observation period. The actuarial survival rate in recipients of hL6 was 0% on day 46, similar to the 0% observed on day 45 in the first experiment. Median survival times (MSTs) were also similar, suggesting that the differences in the present experiment were not related to the severity of the GVHD response in these two experiments.

We compared hCTLA4-Ig at a dose of 100 μg per injection to 250 μg per injection beginning on day −1 pre-BMT and continuing every other day through day 29 post-BMT. An additional group of mice was injected every other day with 100 μg per injection from day −1 through +15 to further minimize potential immune responsiveness (Table 1, experiment 3). Compared with the PBS-treated control group, recipients of hCTLA4-Ig at 250 μg per injection or 100 μg per injection through day 15 post-BMT had a significantly ($P = .019$; $P = .023$, respectively) higher actuarial survival rate, whereas recipients of 100 μg per injection through day 29 did not ($P = .21$). None of the recipients of hCTLA4-Ig survived beyond day 37 post-BMT. Histologic examination was consistent with GVHD in a pattern similar to that observed in experiment 1 described above.

mCTLA4-Ig is not superior to hCTLA4-Ig in preventing lethal GVHD. To further reduce the possibility that hCTLA4-Ig was only partially effective because a xenoge-

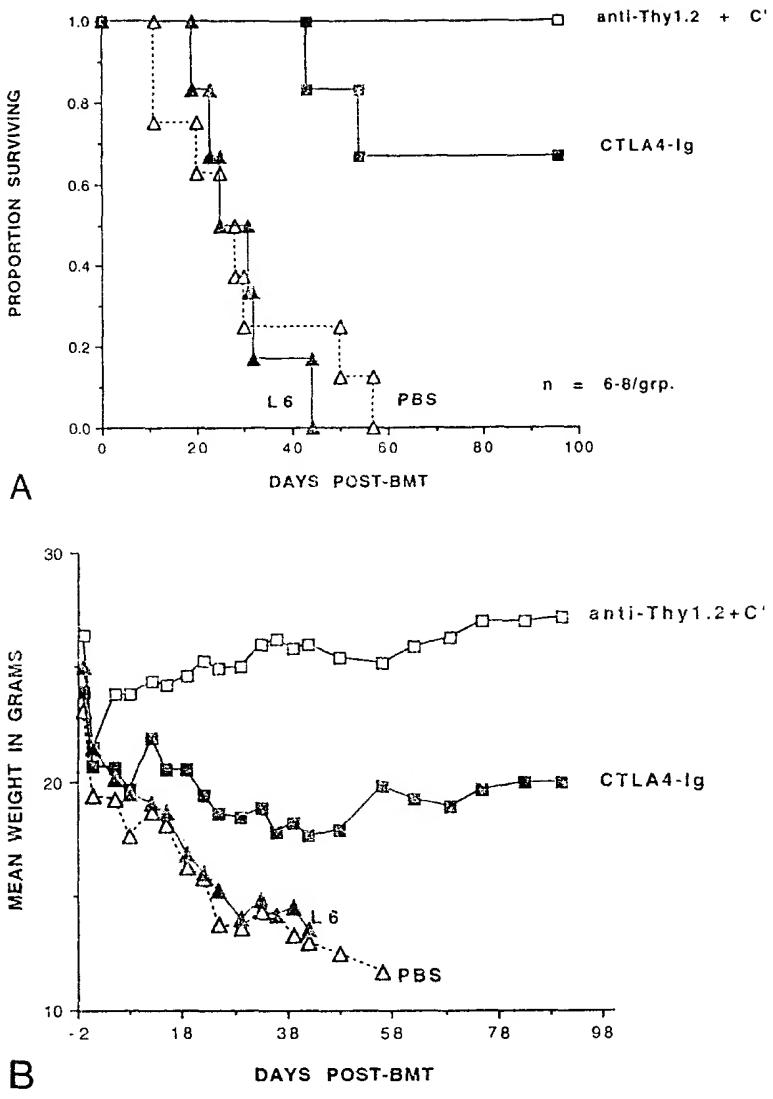

Fig 1. hCTLA4-Ig administered in vivo to lethally irradiated recipients of fully allogeneic BMS grafts significantly reduces GVHD-induced mortality. B10.BR recipients were lethally irradiated and transplanted with 25×10^6 C57BL/6 BM + 25×10^6 C57BL/6 splenocytes. One group received splenocytes that were treated in vitro with anti-Thy1.2 + C'. Mice were injected with hCTLA4-Ig or hL6 (100 μg per injection every other day from days 0 through 25 post-BMT) or PBS. Six (hCTLA4-Ig; hL6) or 8 (PBS; anti-Thy1.2 + C'-treated BMS) mice per group received transplants. (A) Actuarial survival is plotted. (B) Mean weight curves are plotted for the same experiment.

neic protein response could be generated in vivo, we compared the actuarial survival rates of recipients of hCTLA4-Ig to mCTLA4-Ig at a dose of 100 μg per injection days -1 to $+13$ post-BMT and then thrice weekly through day 29 (hCTLA4-Ig) (n = 8 per group) or day 22 (mCTLA4-Ig, because of limited material availability) (n = 5) post-BMT (Table 1, experiment 4). We have previously used this more intensive administration schedule to completely prevent lethal GVHD in this system with anti-CD3ϵF(ab')$_2$ fragments.[57]

Compared with their respective irrelevant control MoAbs, recipients of hCTLA4-Ig or mCTLA4-Ig had a significant (P = .0056; P = .0094, respectively) increase in 83-day actuarial survival rate (0% and 0% v 25% and 0%, respectively) (Table 1, experiment 4). Recipients of hCTLA4-Ig did not have a significantly (P = .49) higher actuarial survival rate than recipients of mCTLA4-Ig. Weight data during the injection period for mCTLA4-Ig initially suggested that mCTLA4-Ig would have a highly efficacious anti-GVHD effect, although once injections were discontinued, mice rapidly developed clinical signs of GVHD (not shown).

Peripheral blood was analyzed on days 15 and 28 post-BMT (Table 3). Compared with recipients of in vitro TCD BMS (GVHD-free control), recipients of PBS had leukopenia caused by a profound lymphopenia and a lower hematocrit value on days 15 post-BMT. Recipients of hCTLA4-Ig, hL6, or mCTLA4-Ig showed similar results along with a relative neutrophilia that has been noted to occur in the early phases of a GVHD reaction.[62] Lymphopenia, anemia, and neutropenia were also observed on day 28 post-BMT in recipients of either hCTLA4-Ig or mCTLA4-Ig.

A kinetic (day 7, 11, 14, and 21 post-BMT) flow cytometry analysis of reconstituting donor splenic T cells and BM was performed on a separate cohort of 16 mice per group (hCTLA4-Ig; hL6; in vitro TCD BMS; PBS) segregated from the survival cohort at the beginning of the experiment (Table 4). Congeneic C57BL/6 donors were used to permit determination of the origin of repopulating cells post-BMT,

Table 1. Effect of CTLA4-Ig on GVHD-Induced Mortality in B10.BR Recipients of C57BL/6 BMS

Exp. No.	In Vivo	No. BMT	In Vivo Dose, Schedule	MST (d)	% Actuarial Survival (d)	*P* Value (*v* L6)*
1	No treatment	8	NA	29	0 (58)	
	Anti-Thy1.2 + C′ in vitro	8	NA	>96	100 (96)	
	hCTLA4-Ig	6	100 μg qod d 0 to 25	>96	67 (96)	.0033
	hL6	6	100 μg qod d 0 to 25	26	0 (45)	
2	No treatment	8	NA	33	0 (39)	
	Anti-Thy1.2 + C′ in vitro	8	NA	>62	100 (62)	
	hCTLA4-Ig	7	250 μg qod d −1 to 25	39	13 (62)	.041
	hL6	8	100 μg qod d −1 to 25	34	0 (46)	
3	PBS	8	d −1 qod to 29	22	0 (29)	
	Anti-Thy1.2 + C′ in vitro	8	NA	>100	100 (100)	
	hCTLA4-Ig	8	100 μg qod d −1 to 29	21	0 (39)	.21
	hCTLA4-Ig	8	250 μg qod d −1 to 29	26	0 (36)	.019
	hCTLA4-Ig	8	100 μg qod d −1 to 15	28	0 (37)	.023
4	PBS	8	−1 to 13+ qod 15-29	29	0 (34)	
	Anti-Thy1.2 + C′ in vitro	8	NA	>83	100 (106)	
	hCTLA4-Ig	8	100 μg qod d −1 to 29	37	13 (106)	.007
	hL6	8	100 μg qod d −1 to 29	25	0 (28)	
	mCTLA4-Ig	8	100 μg qod d −1 to 22	41	0 (61)	.009
	mL6	8	100 μg qod d −1 to 22	22	0 (30)	
5	PBS	8	−1 to 28	28	13 (77)	
	Anti-Thy1.2 + C′ in vitro	8	NA	>46	100 (77)	
	mCTLA4-Ig	8	100 μg d −1 to 28	45	38 (77)	.035
	mL6	8	100 μg d −1 to 28	26	0 (57)	

B10.BR recipient mice were lethally irradiated on day −1 pre-BMT and administered 25 × 10^6 C57BL/6 BM cells + 25 × 10^6 C57BL/6 splenocytes (BMS) on day 0. Recipients were injected intraperitoneally with human CTLA4-Ig (hCTLA4-Ig), mouse CTLA4-Ig (mCTLA4-Ig), irrelevant human or mouse proteins (hL6; mL6), or PBS at the indicated doses and schedules shown above. The number of mice undergoing BMT is listed. *P* values shown were by log-rank analysis of Kaplan-Meier actuarial survival curves. Experiments are shown in sequential order performed.

Abbreviations: qod, every other day; NA, not applicable.

* In experiment 3, *P* value is compared with PBS group.

originating from donor splenocytes (Ly 5.2$^+$), donor BM (Ly 5.1$^+$, H-2^{b+}), or host (Ly 5.1$^+$, H-2^{k+}).

On days 7 (data not shown) and 11 post-BMT, there were significantly higher numbers of mature (Ly 5.2$^+$) donor-derived splenic-localized T cells in recipients of hCTLA4-Ig, hL6, or PBS compared with in vitro TCD BMS. CD4$^+$ and especially CD8$^+$ T cells appeared to be similarly increased in these three groups relative to the in vitro TCD BMS group. Donor (H-2^{b+}) BM-derived (Ly 5.1$^+$) B cells were significantly decreased and myelomonocytic (Mac1$^+$) cells were significantly increased on day 11 post-BMT (data not shown). This was followed on day 21 post-BMT by a state of splenic hypoplasia in these three groups (data not shown). Throughout the study period, only recipients of in vitro TCD BMS had detectable numbers of host cells. There were few ($\leq 2 \times 10^6$ per spleen) NK1.1$^+$ cells, CD4$^+$CD45R$^+$, CD4$^+$CD45R$^-$ or IL-2R$^+$ T cells, Ly5.2$^+$ Mac1$^+$ or B220$^+$ cells, and virtually no detectable TCR$\alpha/\beta^+4^-8^-$ cells in any of the four groups (data not shown).

The most striking aspects of the BM analysis in recipients of PBS, hCTLA4-Ig, or hL6 compared with in vitro TCD BMS were (1) reduced total BM cellularity on day 14, and (2) reduced numbers of donor (H-2^b) Ly 5.1$^+$ B cells with increased numbers of donor Ly 5.2$^+$ T (CD4$^+$ or CD8$^+$) cells and Mac1$^+$ cells present throughout the study period (data not shown). When comparing recipients of hCTLA4-Ig to hL6 or PBS, no significant differences in these parameters were apparent.

Daily IP injections of mCTLA4-Ig do not eliminate lethal GVHD. In experiment 4, mice appeared GVHD-free while receiving mCTLA4-Ig but rapidly developed GVHD after discontinuation of mCTLA4-Ig injections (data not shown). Therefore, we reasoned that a more prolonged injection period might be more efficacious in preventing GVHD. Mice were injected with mCTLA4-Ig or mL6 (100 μg per injection) IP daily beginning on day −1 and continuing through day 28 post-BMT. Eight mice per group (including in vitro TCD BMS; PBS controls) received transplants (Table 1, experiment 5).

Murine recipients of daily mCTLA4-Ig had a significantly ($P = .035$) higher 77-day actuarial survival rate (38% *v* 0%, respectively) compared with recipients of mL6 (Table 1, experiment 5). Mice were not GVHD-free by clinical examination. Twelve days post-BMT, mean body weights were 1.2 g higher in recipients of mCTLA4-Ig compared with mL6. After this period of time, both groups had a comparable daily loss in mean body weights reaching a steady-state nadir at 23 days post-BMT (data not shown). Thus, daily mCTLA4-Ig at a dose of 100 μg per injection IP was not superior in protecting mice against lethal GVHD compared with hCTLA4-Ig historical controls (eg, Table 1, experiment 1).

DISCUSSION

We have shown that in vivo blockade of CD28/CTLA4:B7/BB1 T-cell costimulation with CTLA4-Ig con-

Table 2. Multi-Organ System Phenotypic Analysis of Long-Term Survivors Administered Anti-Thy1.2 + C′–Treated BMS or hCTLA4-Ig In Vivo

Group	Total Counts	H-2k$^+$	H-2b$^+$	B220$^+$	CD3$^+$ CD4$^+$	CD3$^+$ CD8$^+$	TCRα/β^+ CD4$^-$8$^-$	Mac$^+$
	Splenocytes (absolute no.)							
		Host	Donor Cells					
Anti-Thy1.2 + C′	90 (7)	1 (0)	86 (6)	57 (3)	19 (2)	6 (1)	8 (1)	11 (1)
hCTLA4-Ig	65 (7)*	0 (0)†	64 (7)*	26 (10)†	6 (2)†	5 (1)	7 (1)	13 (6)
	Peripheral Blood Mononuclear Cells (%)							
		Host	Donor Cells					
Anti-Thy1.2 + C′	ND	1 (0)	94 (1)	52 (2)	28 (2)	5 (0)	ND	ND
hCTLA4-Ig	ND	0 (0)†	97 (2)	23 (10)†	13 (4)†	11 (5)	ND	ND
	Thymus (absolute no.)							
							CD4$^+$8$^+$	
Anti-Thy1.2 + C′	55 (7)	ND	ND	ND	7 (1)	3 (0)	43 (5)	ND
hCTLA4-Ig	47 (19)	ND	ND	ND	10 (4)	3 (1)	33 (13)	ND
	BM (absolute no.)							
		Host	Donor Cells					
					CD4$^+$ or CD8$^+$			
Anti-Thy1.2 + C′	52 (5)	0 (0)	49 (6)	13 (1)	2 (0)		3 (0)	16 (2)
hCTLA4-Ig	51 (22)	0 (0)	51 (24)	8 (3)	2 (1)		3 (1)	24 (12)

Ninety-six days post-BMT, peripheral blood, splenocytes, BM, and thymocytes were obtained from mice in Fig 1 administered anti-Thy1.2 + C′–treated BMS (n = 6) or nontreated BMS with in vivo hCTLA4-Ig (100 μg IP every other day from days 0 to 25) (n = 4). Two- or three-color fluorescence-activated cell sorter (FACS) analysis was performed as described in Materials and Methods. The numbers in parentheses are values for the standard error of the mean. All values listed are percent (as indicated) or absolute values $\times 10^{-6}$. Zero indicates 0% or $<0.5 \times 10^6$ cells, as appropriate.

Abbreviation: ND, not determined.

* $P < .01 > .05$.

† $P < .05$.

sistently reduces lethal GVHD in murine recipients of fully allogeneic BMS, significantly prolonging survival rates with up to 63% of mice surviving greater than 3 months post-BMT. Prolongation of actuarial survival rates was variable with 0% to 63% of CTLA4-Ig–treated mice surviving the entire observation period. The reasons for the variable effect on survival are unclear. Histologic examination of hCTLA4-Ig–treated recipients dying with clinical signs of GVHD showed evidence of GVHD in the liver, lung, and colon with similar findings as observed in recipients of irrelevant antibody or saline injections. Flow cytometry analysis of long-term survivors showed a B-cell and CD4$^+$ T-cell lymphopenia in the peripheral blood and spleen, also consistent with GVHD in some strain combinations.[62] No significant differences were noted in the central compartments (thymus or BM) suggesting that the findings in the peripheral compartments (spleen and the blood) may reflect an earlier post-BMT period of more active GVHD.

Because GVHD prevention was incomplete, perhaps CTLA4-Ig infusions were less effective in inhibiting the particular T-cell subset responsible for mediating GVHD in this strain combination. Several lines of evidence indicate that both T-cell subsets, CD4$^+$ and CD8$^+$ T cells, contribute to the GVHD process. Depletion of both CD4$^+$ and CD8$^+$ T cells with anti-CD4 + anti-CD8 MoAbs prevents lethal GVHD, whereas in vivo depletion of CD4$^+$ T cells alone is partially effective and depletion of CD8$^+$ T cells alone is marginally beneficial (B.R.B., P.A.T., D.A.V.: unpublished observations, May 1992). The in vivo administration of rapamycin, a macrolide immunosuppressive agent that blocks cytokine signal responsiveness,[63] eliminates lethal GVHD and is associated with a reduction in the expansion of early mature donor splenic-derived CD4$^+$ T cells and later (post-BMT) is associated with a reduction in the number of mature donor splenic-derived CD8$^+$ T cells. In other studies, the presence of CD3ϵ^+CD8$^+$ T cells on day 14 post-BMT appeared to be closely associated with GVHD[57] and in vitro depletion of T cells with cytolytic capacity prevents GVHD.[39,40] Together, these studies suggest that CD4$^+$ T cells drive the expansion of CD8$^+$ T cells and may, especially in the absence of CD8$^+$ T cells, directly participate in the GVHD tissue destructive process.

To be effective for GVHD prevention, CTLA4-Ig infusions must inhibit the expansion of donor CD4$^+$ and CD8$^+$ T cells in this strain combination. Did this occur? Flow cytometry studies showed that CD8$^+$ T-cell repopulation was

Table 3. Effect of CTLA4-Ig on Hematopoietic Reconstitution in B10.BR Recipients of C57BL/6 BMS Grafts

	Day 15				Day 28			
Group	WBC	ANC	ALC	Hct	WBC	ANC	ALC	Hct
Anti-Thy1.2 + C′	6.6 (0.7)	1.3 (0.1)	5.3 (0.7)	48.8 (0.9)	9.8 (0.9)	2.1 (0.6)	7.6 (0.)	47.4 (0.7)
PBS	1.6 (0.3)*	1.1 (0.3)	0.5 (0.1)*	45.6 (0.8)*	1.2 (0.2)*	1.1 (0.2)	0.1 (0.0)	45.3 (2.4)
hCTLA4-Ig	2.7 (0.4)*	2.0 (0.3)*	0.7 (0.1)*	43.1 (1.0)*	1.5 (0.4)*	0.8 (1.0)*	0.7 (0.4)	41.1 (3.6)†
hL6	3.5 (0.5)*	2.6 (0.4)*	0.9 (0.1)*	40.9 (1.3)*	ND	ND	ND	ND
mCTLA4-Ig	2.2 (0.3)*‡	1.5 (0.3)§	0.7 (0.1)*‡	41.3 (1.1)*	1.3 (0.2)*	0.6 (0.1)*	0.6 (0.2)	34.2 (0.5)*
mL6	1.0 (0.3)*	0.6 (0.2)*	0.3 (0.9)*	35.3 (9.5)†	0.8‖	0.7‖	0.1‖	33.0‖

All surviving mice (n = 4 to 8 per group, unless indicated) from Table 1, experiment 4, were analyzed for hematopoietic reconstitution on day 28 post-BMT. Values for WBC, ANC, and ALC are absolute numbers ($\times 10^{-6}/\mu L$); values for Hct are percent. All values are followed by standard error of the mean values in parentheses.

Abbreviations: ND, not determined; WBC, white blood cell count; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; Hct, hematocrit.

* $P < .05$ compared with anti-Thy1.2 + C′–treated BMS.

† $P > .05$ and $< .1$ compared with anti-Thy1.2 + C′-treated BMS.

‡ $P < .05$ compared with the appropriate irrelevant control MoAb.

§ $P > .05$ and $< .1$ as the appropriate irrelevant control MoAb.

‖ Only one mouse remained in this group on day 28 post-BMT.

not inhibited by hCTLA4-Ig injection. $CD8^+$ T cells were the predominant T-cell population at all time periods post-BMT in hCTLA4-Ig–treated mice despite the fact that the donor spleen used to generate GVHD contains approximately twofold more $CD4^+$ compared with $CD8^+$ T cells. Others have shown that costimulation of T cells by B7:CD28/CTLA4 interaction can facilitate the generation of CTLs from resting T cells in vitro[64] or in vivo.[65,66] Mice receiving hCTLA4-Ig, hL6, or PBS had comparable numbers of mature splenic donor-derived $CD8^+$ T cells, each higher than recipients of in vitro anti-Thy1.2+ C′–treated BMS on days 11 and 14 post-BMT.

There was a trend toward greater numbers of $CD4^+$ T cells in these groups compared with in vitro anti-Thy 1.2 + C′–treated BMS. We had anticipated that hCTLA4-Ig would inhibit donor $CD4^+$ T-cell expansion because (1) blockade of B7 binding to CD28/CTLA4 in vitro inhibits murine or human antigen-specific T-helper type 1 (IL-2–producing) clones from recognizing antigen present during blockade[18,24,33,34,67]; (2) CTLA4-Ig infusion in vivo in rats decreases proliferative responses to allogeneic cardiac allografts, even in nontolerant rats[37]; (3) CD28 deletional mice had impaired T-helper cell-dependent facilitation of IgG1 and IgG2b Ig synthesis and lectin-induced mitogenicity[68]; and (4) a significant GVHD protective effect was observed in a $CD4^+$ T-cell–mediated GVHD system when resting host B cells, which do not constitutively express B7, were injected into donors 7 to 11 days before harvesting spleno-

Table 4. Sequential Early Post-BMT Splenic Phenotypic Analysis of Recipients of Anti-Thy1.2 + C′–Treated BMS or hCTLA4-Ig In Vivo

				Ly5.2		
Group	Total Counts	Ly5.1 H-2^b	Ly5.2 H-2^b	$CD3^+$	$CD4^+$	$CD8^+$
			Day 11			
Anti-Thy1.2 + C′	30 (1)	23 (2)	2 (0)	1 (0)	1 (0)	0 (0)
PBS	42 (5)	28 (3)	6 (1)*	5 (1)*	2 (0)†	3 (0)*
hCTLA4-Ig	61 (6)*	43 (3)*	10 (2)*	8 (2)*	2 (0)†	5 (1)*
hL6	43 (8)	32 (7)	7 (1)*	5 (1)*	2 (1)†	3 (0)*
			Day 14			
Anti-Thy1.2 + C′	44 (6)	35 (5)	2 (1)	1 (0)	1 (0)	1 (0)
PBS	43 (10)	28 (7)	10 (3)†	8 (2)†	3 (2)	5 (2)*
hCTLA4-Ig	24 (5)†	17 (4)†	5 (2)	4 (1)	1 (0)	3 (1)†
hL6	15 (4)*	9 (2)*	4 (1)	3 (1)*	1 (1)	2 (1)

Four mice per group from Table 1, experiment 4, were killed at the indicated day post-BMT for two- or three-color FACS analysis of splenocytes as described in Materials and Methods. All values listed absolute values $\times 10^{-6}$. The numbers in parentheses are values for the standard error of the mean.

* $P < .05$ compared with anti-Thy1.2 + C′–treated BMS.

† $P < .1$ and $> .05$ compared with anti-Thy1.2 + C′–treated BMS.

‡ $P < .05$ comparing hCTLA4-Ig with hL6.

§ $P < 0.1$ and $> .05$ comparing hCTLA4-Ig with hL6.

cytes as a source of GVHD-causing T cells.[69] The donor T cells were specifically nonresponsive to host but not third party alloantigens in vitro and in vivo. A lesser effect was seen in a fully allogeneic strain combination, which typically requires participation of $CD4^+$ + $CD8^+$ T cells. We cannot reach definitive conclusions as to whether hCTLA4-Ig injections significantly inhibited $CD4^+$ T-cell repopulation post-BMT because of the relatively small differences in the number of donor $CD4^+$ T cells in the spleens of control recipients of PBS compared with anti-Thy1.2 + C′–treated BMS.

From the flow cytometry data, we do not know if CTLA4-Ig provided an inadequate inhibition of just donor $CD8^+$ T-cell expansion or if both $CD4^+$ and $CD8^+$ T cells were not sufficiently inhibited. Future experiments infusing CTLA4-Ig in GVHD systems that are known to be controlled exclusively by $CD4^{+70,71}$ or $CD8^{+70,71}$ T cells are indicated.

One possible explanation for our findings is that CTLA4-Ig could be less effective in inhibiting primed T cells. In a lethal acute GVHD system, early post-BMT, there is likely a massive priming and expansion of donor cells with alloreactive capabilities that may be less susceptible to CTLA4-Ig inhibition. However, Tan et al[33] have shown that CTLA4-Ig in vitro can block primed human T cells generated in a mixed lymphocyte culture reaction. In addition, Lin et al[38] have recently shown that CTLA4-Ig was more effective in preventing cardiac allograft rejection in rats when administered 2 days after leukocyte priming. Because we have not directly tested whether delayed infusion of CTLA4-Ig after an initial priming period would be more effective than the strategies reported in this manuscript, we are unable to resolve this issue.

We also cannot exclude the possibility that the administration of CTLA4-Ig favored the development of a particular subset of T cells that contributed to the GVHD process. Although blockade of B7:CD28/TLA4 interaction has been shown to induce anergy in $CD4^+$ Th1 cells, $CD4^+$ Th2 (IL-4– or IL-10–producing T-helper cells) cells have not been rendered anergic under similar conditions. Tan et al[33] showed that CTLA4-Ig blockade of primed T cells did not affect IL-4 mRNA accumulation at a time when Th1 cytokine (IL-2 and interferon γ) mRNA accumulation was markedly reduced, suggesting that Th2 cells would be favored in terms of cell outgrowth. Also consistent with the hypothesis are data obtained with in vivo infusion of hCTLA4-Ig, which shows that secondary antibody response, typically facilitated by Th2 cells, is less dramatically affected than primary antibody response.[35] However, Th2 cells have generally been implicated in mediating chronic murine GVHD,[72] and recently investigators have shown that the induction of Th2 cells may be used to decrease acute GVHD.[73] Therefore, we do not favor this explanation for the partial effect of CTLA4-Ig on GVHD prevention.

Because mice with higher CTLA4-Ig sera levels appeared clinically healthier and survived longer than mice with lower levels, we speculated that the amount of hCTLA4-Ig administered in our initial experiments might have been inadequate despite the fact that this amount provided significant in vivo biologic effects in other systems.[35,36] However, neither a 2.5-fold greater amount of CTLA4-Ig protein nor prolongation of the injection schedule improved our initial results. We considered the possibility that hCTLA4-Ig was immunogenic (because of the human Ig fusion partner), suggesting to us that a shorter duration of treatment would decrease immunogenicity and improve efficacy. This was not the case. We also tested whether mCTLA4-Ig, which has been observed to require a lower concentration to inhibit mixed lymphocyte culture responses in vitro, would be more efficacious than hCTLA4-Ig because the $C\gamma1$ fusion partner is mouse rather than human Ig. This did not appear to be the sole explanation because direct comparative studies of each fusion protein did not support this hypothesis. We did not obtain sera for hCTLA4-Ig and mCTLA4-Ig levels in these experiments because mice had significant GVHD early post-BMT in all groups (except those receiving anti-Thy1.2 + C′–treated BMS) and would be at increased risk for adverse events associated with blood withdrawal. Although we do not know if mCTLA4-Ig was less rapidly cleared than hCTLA4-Ig in these experiments, regardless of whether mCTLA4-Ig persisted longer in the circulation, mCTLA4-Ig was not more advantageous for survival than hCTLA4-Ig in our studies.

These data differ somewhat to those obtained in lethally irradiated B6D2 (C57BL/6 × DBA/2:H-$2^{d/b}$) F1 recipients of C57BL/6 allografts in which mCTLA4-Ig was more effective than hCTLA4-Ig. Consistent with our data, actuarial survival rates of CTLA4-Ig–treated recipients were also variable. Up to 40% to 60% of recipients of mCTLA4-Ig surviving long term (≥4 months post-BMT)[74] had evidence of GVHD as manifested by B lymphopenia, T-cell immunodeficiency, and a pulmonary lymphocytic infiltration consistent with a sublethal GVHD syndrome.[57] Even though CTLA4-Ig binds to activated B cells, CTLA4-Ig infusion is unlikely to directly result in depletion of mature B cells in either our studies or those of Wallace et al[74] because, in murine recipients of repeated sheep RBC immunizations, primed B cells are not inhibited from producing antisheep RBC antibody by CTLA4-Ig administration.[35] The overall similar results obtained in two distinct donor-recipient strain combinations differing by one (C57BL/6 transplanted into B6D2 F1) or two (C57BL/6 transplanted into B10.BR) MHC haplotypes show that the anti-GVHD effect of CTLA4-Ig is not entirely strain dependent.

Because CTLA4-Ig blocks T-cell costimulation and T cells can facilitate hematopoietic recovery post-BMT, we were interested in discerning if CTLA4-Ig would inhibit early post-BMT hematopoietic reconstitution. Neither hCTLA4-Ig nor mCTLA4-Ig interfered with hematopoietic recovery 15 days post-BMT. Hematopoietic parameters in CTLA4-Ig–treated mice were consistent with GVHD and included neutrophilia, lymphopenia, and lower hematocrit values.[62] In hCTLA4-Ig– or mCTLA4-Ig–treated B6D2 F1 recipients of C57BL/6 allografts, a reduction in early (day 7) and long-term (>100 days) post-BMT peripheral blood leukocytes was observed, which did not occur in syngeneic recipients of hCTLA4-Ig, suggesting that the leukopenia was a reflection of sublethal GVHD.[74]

In summary, we have shown that CTLA4-Ig consistently and significantly reduces lethal GVHD in murine recipients

of fully allogeneic donor cells without interfering with hematopoietic recovery post-BMT. Prolongation of actuarial survival rates by CTLA4-Ig is variable and does not appear to be related to the dose, schedule, or species (human or mouse) of CTLA4-Ig. Surviving recipients have clinical, laboratory, and histologic evidence of GVHD that we hypothesize is related, at least in part, to the relative inefficiency of inhibition of expansion of mature donor spleen-derived $CD8^+$ T cells, the final mediators of GVHD in this strain combination. Experiments combining CTLA4-Ig administration with other agents that block costimulatory capacity or partially inhibit T-cell function may further improve the anti-GVHD efficacy of CTLA4-Ig and permit the in vivo induction of host alloantigen nonresponsiveness.

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response to distinguish differences among the human islet donors also supports the direct recognition of the polymorphic MHC products expressed on the human islet cells. The future of immunosuppressive therapies in transplantation and autoimmune disease depends on their ability to induce long-term, antigen-specific unresponsiveness. The capacity of CTLA4Ig to significantly prolong human islet graft survival in mice in a donor-specific manner suggests that blocking the interaction of costimulatory molecules such as CD28-B7 may provide an approach to immunosuppression.

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Immunosuppression in Vivo by a Soluble Form of the CTLA-4 T Cell Activation Molecule

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In vitro, when the B7 molecule on the surface of antigen-presenting cells binds to the T cell surface molecules CD28 and CTLA-4, a costimulatory signal for T cell activation is generated. CTLA4Ig is a soluble form of the extracellular domain of CTLA-4 and binds B7 with high avidity. CTLA4Ig treatment in vivo suppressed T cell–dependent antibody responses to sheep erythrocytes or keyhole limpet hemocyanin. Large doses of CTLA4Ig suppressed responses to a second immunization. Thus, costimulation by B7 is important for humoral immune responses in vivo, and interference with costimulation may be useful for treatment of antibody-mediated autoimmune disease.

Costimulatory signals delivered by antigen-presenting cells (APCs) have been proposed to control immune responses to transplanted tissues (*1*). Antigenic stimulation of T cells in vitro in the absence of costimulation leads to aborted T cell proliferation and the development of functional unresponsiveness or clonal anergy of T cells (*2*). Several molecules on APCs augment T cell proliferation (*3*, *4*) and regulate functional unresponsiveness in vitro (*4*). The B7 activation molecule binds CD28 (*5*) and delivers a costimulatory signal for T cell proliferation (*6*). T cell–dependent B cell differentiation requires the interaction of B7 with CD28 (*7*). CTLA-4, a T cell molecule homologous to CD28 (*8*), also binds the B7 counter-receptor (*9*). CTLA4Ig, a chimeric immunoglobulin C_γ fusion of CTLA-4, binds with high avidity (dissociation constant ~12 nM) to B7 and potently blocks T cell–dependent immune responses in vitro (*9*). CD28 probably participates in costimulation required to prevent anergy induction in T cell clones (*10*), in unresponsiveness in human mixed lymphocyte reactions (*11*), and in the costimulation of antigen-specific interleukin-2 production of human T cells (*12*). Despite data that indicate the importance of B7-CD28 interactions in the costimulation of in vitro T cell responses, the role of these interactions in regulating in vivo immune responses is unknown. Here, we show that CTLA4Ig is a potent suppressor of antibody responses in vivo.

Human CTLA4Ig [human CTLA-4 and human immunoglobulin (Ig)] binds to murine B7 and inhibits murine T cell responses in vitro (*13*). These findings led us to test the effects of human CTLA4Ig on murine immune responses in vivo. CTLA4Ig was purified to homogeneity by protein A chromatography from a serum-free culture medium of transfected mammalian cells (*14*). The chimeric monoclonal antibody (MAb) L6, which has a murine region and a human Fc region, was used as a control.

We first measured serum clearance of human CTLA4Ig in mice (Fig. 1). A plot of serum CTLA4Ig levels versus time was biphasic, giving a time of half-clearance ($t_{1/2}$) of ~4 hours and ~30 hours for the two components. Serum clearance after multiple injections of CTLA4Ig was more complex and appeared dose-related. The $t_{1/2}$ for the more slowly clearing component was increased to ~4 days after six daily intravenous injections of CTLA4Ig (200 μg per injection), and functionally active CTLA4Ig was detected in mouse serum for up to ~5 weeks after the last treatment with CTLA4Ig. No overt toxicity of CTLA4Ig was noted.

The ability of CTLA4Ig to suppress for-

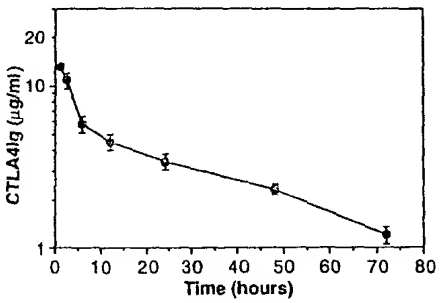

Fig. 1. Serum clearance of human CTLA4Ig in mice. BALB/c mice were each given a single intravenous injection of 50 μg of CTLA4Ig prepared from COS cells. At the indicated times, the mice were bled retro-orbitally. The binding of functional CTLA4Ig from sera to B7$^+$ CHO cells was measured by flow cytometry (*6*). CTLA4Ig concentrations were quantitated by comparison of the degree of binding with the binding of known concentrations of CTLA4Ig. Values represent mean concentrations ± SD from five mice.

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mation of plaque-forming cells (PFCs) that produce antibodies (Abs) to sheep red blood cells (SRBCs) was examined. CTLA4Ig or MAb L6 was administered daily by intravenous injection beginning immediately after administration of the SRBCs and continuing for 2 days. SRBC-specific PFCs were measured on day 14 after immunization (Fig. 2). PFC formation was suppressed in CTLA4Ig-treated mice in a dose-dependent manner. CTLA4Ig suppressed the PFC response by >50% with as little as 1 μg per injection and completely inhibited the response with 75 μg per injection. Administration of up to 100 μg per injection of chimeric MAb L6 did not significantly affect PFC formation, which indicates that suppression by CTLA4Ig was not a result of the Fc portion of the molecule.

We also examined the ability of CTLA4Ig to suppress Ab responses to a soluble protein antigen, keyhole limpet hemocyanin (KLH) (Fig. 3). Administration of CTLA4Ig after immunization suppressed Ab response to KLH. Maximal suppression was observed with a 3-day treatment with CTLA4Ig beginning immediately after immunization and continuing for 2 days thereafter (days 0 to 2). Essentially identical suppression was seen if the start of CTLA4Ig administration was delayed for up to 2 days. These data indicate that B7 is not required for up to 2 days after immunization with KLH. Thus, B7-CD28 interactions are most likely to maintain or amplify an immune response rather than initiate it (5). Further delay in starting CTLA4Ig treatment resulted in less effective suppression; when treatment was started on day 4 or 5, partial suppression of Ab production was observed. This may indicate that B7-CD28 interactions also function at later stages of an immune response.

The effect of CTLA4Ig on secondary Ab responses was examined. The mice were immunized with KLH, and on day 20 received a secondary immunization with KLH and treatment with CTLA4Ig or chimeric L6 (50 μg per mouse for 6 days). Serum titers of Abs to KLH rose from 2,600 ± 630 (reciprocal dilutions ± SD) on day 20 to 93,000 ± 29,000 on day 31 in chimeric L6-treated mice; in CTLA4Ig-treated mice, titers rose from 2,400 ± 100 to 23,000 ± 9,800. Thus, CTLA4Ig treatment could suppress secondary Ab responses to KLH, although less effectively than it suppressed primary responses.

Experiments were performed to elucidate the mechanism of immunosuppression by CTLA4Ig. Spleens removed on day 4 from mice immunized with SRBCs with or without chimeric L6 treatment were markedly enlarged. In contrast, spleens from mice immunized with SRBCs and then treated for 3 days with CTLA4Ig (>~50 μg per injection) were of normal size. Cell yields of spleens from SRBC-immunized mice, with or without MAb L6 treatment, were ~1.6 times greater than of spleens from naïve and CTLA4Ig-treated mice. Thus, splenomegaly most likely resulted from antigen-induced lymphoproliferation. The relative numbers of T cells and total major histocompatibility complex (MHC) class II antigen-positive cells (B cells, monocytes, and dendritic cells) in treated and untreated mice were examined by flow cytometry (15). Splenocytes from naïve mice, mice treated with SRBCs plus chimeric L6, and mice treated with SRBCs plus CTLA4Ig were 45 ± 5%, 43 ± 4%, and 43 ± 7% positive for Thy-1.2, and 34 ± 5%, 38 ± 6%, and 34 ± 4% MHC class II–positive, respectively. Splenocytes isolated from mice treated with SRBCs and chimeric L6 or CTLA4Ig on day 4 had similar B7 expression (measured by CTLA4Ig binding) and also similar induction of B7 expression as shown by overnight incubation in vitro with lipopolysaccharide (5). Spleen B cells from SRBC- and CTLA4Ig-treated animals on day 4 did not show decreased ability to mobilize Ca^{2+} after surface Ig was cross-linked. Taken together, these results suggest that CTLA4Ig treatment did not result in gross depletions or changes in responsiveness of spleen B cells.

In vivo immunosuppression by CTLA4Ig was associated with altered T cell responses (Fig. 4). Proliferative responses were measured for spleen cells from naïve mice and from mice immunized in vivo with SRBCs and treated with chimeric L6 or CTLA4Ig. Splenocytes from SRBC-im-

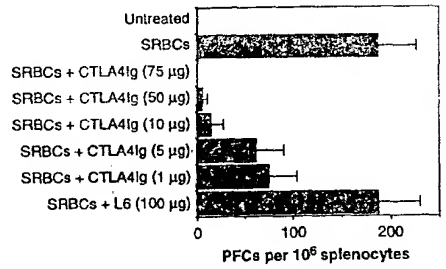

Fig. 2. CTLA4Ig suppresses in vivo induction of SRBC-specific plaque-forming cells. BDF_1 (C57BL/6 × DBA/2)F_1 mice 6 to 8 weeks of age were left untreated or immunized by intravenous injection with 5 × 10^7 SRBCs and then treated with three daily intravenous injections of the indicated amounts of CTLA4Ig or 100 μg of the chimeric MAb L6. After 14 days, spleen cells were assayed for SRBC-specific PFCs by indirect assay with the use of the method of Jerne *et al.* (*23*). Values represent mean ± SD for five mice per group. Similar results were observed when direct PFCs were measured at day 4 after injection. Suppression of PFC formation by CTLA4Ig treatment in vivo was observed in four independent experiments.

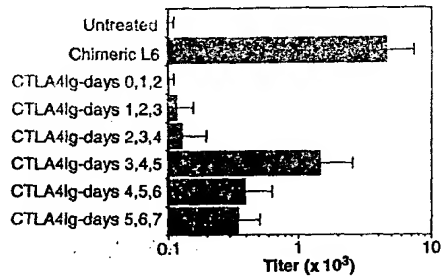

Fig. 3. CTLA4Ig suppresses primary Ab responses to KLH. BALB/c mice were left untreated or immunized on day 0 by intraperitoneal injection of 250 μg of KLH without adjuvant. The mice were then treated by intravenous (tail vein) injection of chimeric MAb L6 (50 μg per day) from days 0 to 7 or by three daily injections on the indicated days with CTLA4Ig (50 μg per injection). Abs to KLH were measured by enzyme-linked immunosorbent assay (ELISA) (*24*) on day 10. Values are expressed as mean titers (± SD) from five individual mice. Similar results were observed in another independent experiment. Suppression of Ab responses to KLH by CTLA4Ig treatment in vivo was observed in four independent experiments.

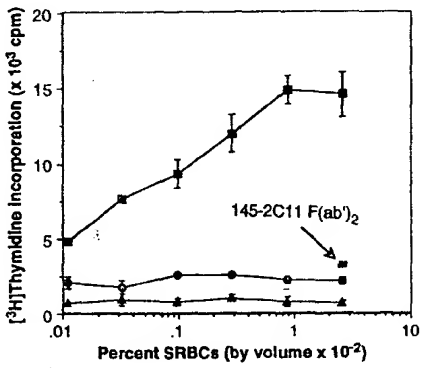

Fig. 4. Splenocytes from CTLA4Ig-treated mice show reduced antigen-specific T cell responses in vitro. BALB/c mice were left untreated (triangles) or were immunized with SRBCs (1 × 10^8 intravenously) and then treated for 3 days with CTLA4Ig (circles) or chimeric MAb L6 (squares) (50 μg per animal per day intravenously). Splenocytes were isolated on day 19 and were cultured at 2 × 10^6 cells/ml in RPMI with 10% fetal bovine serum that contained the indicated concentrations of SRBCs. Cellular proliferation was measured by addition of [³H]thymidine (1 μCi/well) during the final 18 hours of a 3-day culture. Where indicated, a $F(ab')_2$ fragment of the MAb 145-2C11 (to murine CD3) (*16*) was added to a final concentration of 5 μg/ml at the beginning of the culture. Similar results were obtained in two stimulation experiments with a total of five treated and four untreated mice.

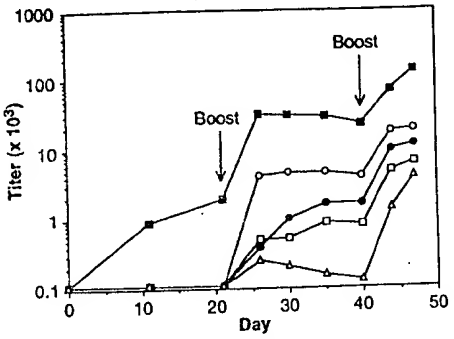

Fig. 5. Immunosuppression by CTLA4Ig is prolonged but not permanent. BALB/c mice were treated with nothing (black circles) or immunized with SRBCs plus L6 (200 μg) (black squares), CTLA4Ig (50 μg) (white circles), CTLA4Ig (100 μg) (white squares), or CTLA4Ig (200 μg) (white triangles). Treatment with L6 or CTLA4Ig was continued for 6 days. The mice were bled at the indicated times, and serum concentrations of Abs to SRBCs were measured by ELISA (*24*). All mice received additional injections of SRBCs on days 21 and 40. Values represent titers of pooled sera from five mice at each time point. Values for mice treated with SRBCs only were identical to those for mice treated with L6 plus SRBCs and have been omitted. Arrows indicate times of secondary and tertiary injections of SRBCs.

munized, chimeric L6-treated animals gave proliferative responses that were dose-dependent with respect to SRBCs; proliferation was blocked by $F(ab')_2$ fragments of a MAb to the T cell receptor complex (*16*). Thus, proliferation was T cell–dependent. Splenocytes from SRBC-immunized, CTLA4Ig-treated animals showed reduced T cell–dependent responses to SRBCs. In other experiments, splenocytes from SRBC-immunized, CTLA4Ig-treated animals showed normal responsiveness to concanavalin A. CTLA4Ig-treated animals also responded normally to allostimulation and to activation by a MAb to CD3 (*17*). These findings are consistent with previous studies (*9*, *13*) showing that CTLA4Ig blocks APC-induced T cell proliferation in vitro and suggest that inhibition of SRBC-specific immune responses in vivo resulted at least in part from reduced antigen-specific T cell function.

We determined the duration of the immune suppression that was induced by CTLA4Ig treatment after primary immunization (Fig. 5). In this experiment, treatment with CTLA4Ig or chimeric L6 was continued for 6 days after immunization. Treatment with all doses of CTLA4Ig suppressed primary SRBC IgG1 Ab responses in pooled sera by >95%. Secondary Ab responses varied according to the dose of CTLA4Ig. Mice treated with 200 μg of CTLA4Ig showed peak Ab responses that were suppressed ~80% in comparison to those of mice receiving a primary immunization and treated with chimeric L6 Mab. Similar results were obtained when IgM- and IgG2a-specific second-step conjugates were used. Mice treated with 100 μg of CTLA4Ig did not show suppressed secondary responses, and those treated with 50 μg of CTLA4Ig gave accelerated responses. Identical conclusions were reached when Ab levels in individual animals on days 25 and 30 were determined. The group treated with 50 μg of CTLA4Ig showed the greatest variation; three of five mice gave enhanced primary responses, and two mice gave titers within the primary response range. This suggests that some mice from this group exhibited immunological memory even though their primary immune responses were substantially blocked. The mice were reimmunized on day 40 with both SRBCs and KLH. SRBC responses for all groups increased with similar kinetics (Fig. 5), and KLH responses were identical (peak titers ~10,000).

Thus, treatment with large doses (200 μg) of CTLA4Ig led to prolonged immunosuppression but not permanent tolerance of SRBCs. This was also the case with mice whose primary Ab responses to KLH were inhibited by CTLA4Ig (50 μg for 3 days) (Fig. 3). We measured the amount of functional CTLA4Ig in sera from BALB/c mice (Fig. 5) by binding $B7^+$ to Chinese hamster ovary (CHO) cells. Animals treated with 50-, 100-, and 200-μg doses of CTLA4Ig had serum CTLA4Ig concentrations on day 21 of 1, 5, and 10 μg/ml, respectively. By the time the mice were reimmunized on day 40, serum CTLA4Ig levels in all groups had dropped to <2 μg/ml. These data suggest that prolonged immunosuppression was associated with the continued presence of CTLA4Ig in serum.

Recently, much attention (*10*–*12*) has been given to how CD28-B7 interactions provide the second or costimulatory signal required to maintain T cell responsiveness (*2*) in vitro. Support for the involvement of these interactions in regulation of in vivo tolerance is given by the findings of Lenschow *et al.* (*18*), who show that CTLA4Ig treatment induces long-term survival of pancreatic islet cell xenografts. There are several possible explanations why we did not induce tolerance in our studies. SRBCs and KLH are extremely potent immunogens, and inducing tolerance to them may require greater blocking of B7 or additional blocking of other costimulatory molecules (*19*). It is also possible that certain T cell populations vary in their dependence on B7 costimulation for maintained responsiveness. CTLA4Ig treatment may favor the development of interleukin-4–producing Th2 cells, which are refractory to tolerization (*20*). Finally, our results may indicate that the binding of human CTLA4Ig to murine B7 is suboptimal for induction of tolerance. Despite these possibilities, our data suggest that virtually complete suppression (>95%) of in vivo immune responses by CTLA4Ig does not necessarily lead to tolerance.

We demonstrated that CTLA4Ig is a potent immunosuppressive agent in vivo, in agreement with previous in vitro results (*9*, *13*). Our data suggest that CTLA4Ig has attractive features for an immunosuppressive drug (that is, in vivo stability, low toxicity, and high specificity). Immune regulation and tolerance induction have been achieved by administration of MAbs to T cell molecules involved in signal transduction (*21*) or combinations of MAbs that block intercellular adhesion of lymphocytes (*22*). Immunosuppression by CTLA4Ig has shown promise as an approach to manipulate immunity to transplants (*18*). Our results showing that CTLA4Ig also suppressed humoral responses suggest potential uses of CTLA4Ig in the treatment of Ab-mediated autoimmune diseases.

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14. Initially, CTLA4Ig was produced by transient transfection of COS cells as described (*9*). Unless otherwise indicated, experiments were done with CTLA4Ig produced in stably transfected CHO cells. CTLA4Ig cDNA (*9*) was cloned into the mammalian expression vector πLN (provided by A. Aruffo) and cotransfected with the pSV2dhfr selectable marker [S. Subramani *et al.*, *Mol. Cell. Biol.* 1, 854 (1981)] into dhfr⁻ CHO cells. CTLA4Ig-secreting transfectants were isolated and amplified by growth in increasing concentrations of methotrexate. An amplified cell line producing 3 to 10 mg/liter was isolated. These experiments were done with CTLA4Ig preparations that contained endotoxin at <~1.5 U/mg.
15. BALB/c mice were immunized with SRBCs and then treated for 3 days with CTLA4Ig or chimeric L6 as described (Fig. 2). Splenocytes were isolated on day 4, stained with biotinylated MAb to

Thy-1.2 (30-H12) or with biotinylated MAb to I-A^d (AMS-32.1) (PharMingen) followed by fluorescein isothiocyanate–conjugated streptavidin, and analyzed by flow cytometry.
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24. Microtiter wells (Immulon 2; Dynatech) were coated with KLH or a 0.5% octyl glucoside extract of SRBC ghosts. Wells were blocked for 1 hour with sample diluent (Genetic Systems, Seattle) and then washed with phosphate-buffered saline that contained 0.05% Tween 20. Sera were serially diluted in sample diluent and incubated with coated wells for 1 hour at 23°C. Wells were washed, and Ab binding was detected by addition of horseradish peroxidase–conjugated Abs to murine IgG1 (Southern Biotech, Birmingham, AL), then detected with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Genetic Systems). Absorbances at 450 nm (A_{450}) were recorded on microtiter plate reader (Genetic Systems). Titers were determined from dilution curves as the dilution required to give a value for A_{450} of five times background.
25. We thank J. Bradshaw for help with the Ca^{2+} mobilization assays; D. Hewgill for flow cytometry assistance; P. Fell for MAb L6; L. Knox, B. Grauerholz, R. Drager, L. Paul, and other members of the CTLA4Ig working group for help in preparation of CTLA4Ig; and J. Bluestone, K. E. Hellstrom, and A. Aruffo for critical reviews of the manuscript.

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Activation-Induced Ubiquitination of the T Cell Antigen Receptor

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The ζ subunit of the T cell antigen receptor (TCR) exists primarily as a disulfide-linked homodimer. This receptor subunit is important in TCR-mediated signal transduction and is a substrate for a TCR-activated protein tyrosine kinase. The ζ chain was found to undergo ubiquitination in response to receptor engagement. This posttranslational modification occurred in normal T cells and tumor lines. Both nonphosphorylated and phosphorylated ζ molecules were modified, and at least one other TCR subunit, CD3 δ, was also ubiquitinated after activation of the receptor. These findings suggest an expanded role for ubiquitination in transmembrane receptor function.

The TCR is a multicomponent transmembrane receptor that consists of clonally derived heterodimeric antigen recognition elements and a set of invariant subunits. These invariant subunits include the members of the CD3 complex (δ, ε, and γ) and the ζ subunit (*1*). The ζ subunit exists primarily as a disulfide-linked homodimer consisting of two 16-kD monomers (*2*, *3*). A subset of ζ chains undergoes multiple tyrosine phosphorylations upon receptor stimulation such that the predominant form migrates with an apparent molecular size of 21 kD in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*4*–*7*).

To evaluate activation-dependent changes in the migration of ζ, we incubated the T cell hybridoma 2B4 (*8*) in the presence or absence of an activating monoclonal antibody to the CD3 ε subunit of the TCR, 2C11 (*9*). TCRs were immunoprecipitated, resolved on two-dimensional nonreducing-reducing SDS-PAGE (diagonal gels), and immunoblotted with antibodies directed to ζ. In unactivated cells (Fig. 1A), the ζ homodimer appeared as a prominent 16-kD species that migrated at 32 kD before reduction. After activation (Fig. 1B), the 21-kD form of phosphorylated ζ was seen directly above ζ as described (*4*, *5*). Unexpectedly, a number of additional immunoreactive species of 24, 32, and 40 kD were also observed in a variety of disulfide-linked combinations. Less prominent forms with apparent molecular sizes of approximately 27 kD under reducing conditions were also observed.

These larger immunoreactive species were further characterized on two-dimensional gels in which nonequilibrium pH gradient electrophoresis (NEPHGE) under reducing conditions was followed by SDS-PAGE in the second dimension (NEPHGE-PAGE) (Fig. 1, C and D). This demonstrated the activation-specific appearance of a ladder-like group of species above ζ, which corresponded to the 24-, 32-, and 40-kD forms seen on the diagonal gels. At each of these molecular sizes, the proteins were homogeneous with regard to isoelectric point (pI). The 21-kD phosphorylated form of ζ migrated with a more acidic pI. A 27-kD activation-specific acidic form was also seen migrating above the 21-kD form of phosphorylated ζ. On longer exposures of the autoradiograms (*10*), other spots were seen above the 27-kD species, which gave the appearance of a second, less intense ladder-like set of species that converged with the more prominent forms toward a neutral pH. This suggested the modification of ζ and, to a lesser extent, phosphorylated ζ with multimers of a neutral 8-kD protein.

Normal murine splenocytes were next analyzed. Freshly isolated splenocytes (Fig. 2A) and cells that had been incubated at 37°C for

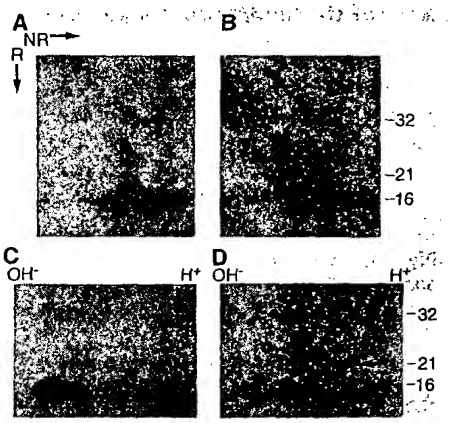

Fig. 1. Activation-induced modifications of ζ in 2B4 hybridoma cells. The 2B4 cells (10^8 cells per condition) and LK cells (an Fc receptor–bearing B cell hybridoma) (*20*) were incubated at 37°C for 45 min in the absence (**A** and **C**) or presence (**B** and **D**) of 2C11 as described (*7*). Cells were chilled to 4°C with phosphate-buffered saline and phosphatase inhibitors (*21*). Cell pellets were lysed in lysis buffer that contained Triton X-100, protease and phosphatase inhibitors (*21*), and postnuclear supernatants immunoprecipitated with a monoclonal antibody directed against the α subunit on 2B4 cells, A2B4-2 (*2*, *22*). Immunoprecipitates were separated under nonreducing conditions in SDS-PAGE tube gels (10.5%) (A and B) or run in NEPHGE tubes (*2*) with a pH range from 3 to 10 (C and D). After equilibration, all tubes were run on SDS-PAGE 12.5% gels. After transfer to nitrocellulose membranes, proteins were immunoblotted with affinity-purified anti-ζ antibodies (551) and detected by ^{125}I-labeled protein A (ICN) (*23*). The 21-kD form of phosphorylated ζ is indicated by arrows (B and D). The spot directly above the arrow in (D) is a hyperphosphorylated form of ζ. Antiserum 551 was raised to a peptide that corresponded to amino acids 151 to 164 of murine ζ (*23*) and does not recognize the η alternative splice of ζ. The diagonal gels (A and B) and the NEPHGE gels (C and D) are from separate experiments. NR, nonreducing; R, reducing. Molecular size markers are indicated to the right in kilodaltons.

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Appendix F

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TRANSPLANTATION
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INHIBITION OF TRANSPLANT REJECTION FOLLOWING TREATMENT WITH ANTI-B7-2 AND ANTI-B7-1 ANTIBODIES

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Antigen-specific T cell activation depends initially on the interaction of the T cell receptor (TCR) with peptide/MHC. In addition, a costimulatory signal, mediated by distinct cell surface accessory molecules, is required for complete T cell activation leading to lymphokine production and proliferation. CD28 has been implicated as the major receptor on T cells responsible for delivering the costimulatory signal. Although two distinct ligands for CD28, B7-1 and B7-2, have been identified on antigen-presenting cells (APC), the costimulatory role of each molecule during a physiological immune response remains unresolved. In the present study, the relative roles of B7-1 and B7-2 interactions were evaluated in an allogeneic pancreatic islet transplant setting. In isolation, anti-B7-2 mAbs and, to a much lesser degree, anti-B7-1 mAbs suppressed T cell proliferative responses to allogeneic islets or splenic APC in vitro. Maximal inhibition of the allogeneic response was observed using a combination of the anti-B7-1 and anti-B7-2 mAbs. Administration of anti-B7-2 but not anti-B7-1 mAbs prolonged C3H allograft survival in B6 recipients, with a combination of both mAbs significantly prolonging rejection beyond either mAb alone. The immunosuppressive effects of the in vivo mAb treatment were not manifested in in vitro analyses as T cells isolated from suppressed mice responded normally to allogeneic stimuli in terms of both proliferation and lymphokine production. However, combined mAb therapy in vivo selectively delayed CD4$^+$ T lymphocyte infiltration into the graft. These data suggest that both B7-1 and B7-2 costimulatory molecules are active in vivo, although B7-2 plays a clearly dominant role in this allograft model. The mechanism of immune suppression in vivo remains unresolved but may occur at sites distinct from the allograft.

Effective T cell activation requires two independent signals. One signal is mediated by engagement of the T cell receptor complex (TCR)* with antigenic peptide bound to major histocompatibility complex class I or class II glycoproteins (Ag/MHC) (*1*). The second signal is mediated by one or more "costimulatory" interactions between activated T cells and antigen-presenting cells (APC) (*2*). TCR signaling in the presence of costimulation results in clonal expansion, production of lymphokines and differentiation of T cells to effector populations. In contrast, TCR signaling in the absence of costimulation leads to a state of induced unresponsiveness (termed anergy) or, in some instances, cell death (*3–7*). Recent studies have suggested that engagement of the CD28 molecule on T cells with ligand on APCs can furnish the costimulatory signal by inducing and maximizing the T cells' ability to produce IL-2, a critical autocrine growth factor (*8–11*). Blocking the engagement of CD28 on T helper cell clones with its ligand on the APCs results in T cell anergy. Furthermore, anti-CD28 mAbs can prevent this anergy induction (*11*).

Previous studies suggested that the relevant costimulatory ligand on activated B cells and dendritic cells was a cell surface glycoprotein, termed B7-1 (B7) (*12–14*). Linsley and coworkers showed that antibodies to B7-1 blocked adhesion of B cells to CD28-transfected CHO cells and that B7-1-transfectants could provide costimulatory signals to the antigen or mitogen-activated T cells (*15*). Furthermore, antihuman B7-1 mAbs were shown to block an allogeneic T cell proliferative response to EBV-transformed B cells (*16*). Finally, CTLA4Ig, a soluble form of the CD28 homolog (CTLA-4), inhibited a variety of immunological responses (*17–22*). CTLA4Ig blocked allo- and xenoantigen responses in vitro in a CD28-dependent manner (*19, 21*), delayed allogeneic transplant rejection in rats (*18–22*), and induced long-term, antigen-specific unresponsiveness in vivo in a xenogeneic human islet transplant model in mice (*19*) and a rat allogeneic heart transplant model when combined with a donor-specific transfusion (*20*).

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* Abbreviations: APC, antigen-presenting cell; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MST, mean survival time; TCR, T cell receptor.

Despite the importance of B7-1 as a CD28 ligand, we and others have recently identified an additional member of the B7 family of molecules, B7-2 (*23–25*). The B7-2 molecule was first uncovered based on the inability of anti-B7-1 mAbs to significantly block allogeneic mixed lymphocyte reactions (MLR) in vitro (*23*). Moreover, the anti-B7-1 mAbs were unable to block CTLA4Ig binding to activated B cells and dendritic cells, consistent with the observation that activated B cells isolated from a B7-1-deficient "knock-out" mouse bound CTLA4Ig and provided T cell costimulatory activity in vitro (*24*). The cloning of the human and mouse B7-2 genes by Freeman et. al. (*26, 27*) and Azuma et al. (*28*) (also termed B70 or CD86) provided direct evidence for the existence of a novel CD28 ligand. B7-2 is a highly glycosylated protein approximately 60–90,000 kDa with stretches of high homology to B7-1 (*25*). The B7-2 molecule binds both CD28 and CTLA-4 and, like B7-1, is expressed constitutively on dendritic cells (*23*). However, unlike B7-1, B7-2 is rapidly (within 6 hr) upregulated following B cell activation through the immunoglobulin receptor (*29*). Together, anti-B7-1 and anti-B7-2 mAbs block the binding of CTLA4Ig to activated B cells and dendritic cells (*29*). Most important, B7-2 appears to play a critical functional role in T cell activation, in vitro. B7-2 transfectants costimulate antigen-specific T cell responses (*26*) and anti-B7-2 mAb, GL-1, inhibits antigen-specific T cell responses (*25*). While in vitro studies indicate that B7-2 dominates the immune response, recent in vivo studies in two autoimmune models suggest that B7-1 and B7-2 play distinct roles, and these roles may be altered by the status of the immune response (*30, 31*). Thus, both B7-2 and B7-1 are potentially important costimulatory molecules capable of initiating T cell responses.

The present study was designed to examine the role of B7-1 and B7-2 during an allogeneic transplant rejection response in vivo. The results demonstrate that anti-B7-2 mAb, either alone or in association with anti-B7-1 mAb, suppresses alloreactivity in vitro and in vivo. Thus, B7-2 provides a dominant costimulatory activity when alloantigen is presented in the context of natural APCs present in splenic cell or islet cell preparations.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). C3H/HeN and BALB/c mice were obtained from the Frederick Cancer Research Facility. All mice were housed in a specific pathogen–free animal barrier facility at the University of Chicago.

Antibodies and recombinant proteins. The hCTLA4Ig, control human Ig, Fab fragments of anti-CD28 (37.51) (*32*), and 16-10A1 (a hamster anti-B7-1 mAb) (*12*) were provided by the Repligen Corporation (Cambridge, MA). The coding sequence for the extracellular portion of human CTLA-4 was joined to the hinge-CH2-CH3 domains derived from a human genomic IgG1 gene by PCR as previously described (*30*). Antibodies and recombinant proteins were purified by binding to immobilized protein A (IPA-300, Repligen), followed by elution with 0.1 M sodium citrate, pH 3.0, and neutralized immediately with 0.1 M Tris base. Antimurine B7-2 mAb, GL-1, and an isotype-matched control rat Ig were prepared as previously described (*25*). All mAbs and soluble receptors were analyzed for binding specificity based on staining of B7-1 and B7-2 transfectants.

Transfectants. Chinese hamster ovary (CHO) cells expressing the murine MHC class II (I-A^d) cDNA were transfected with either murine B7-1/Neo (CHO-Ad/B7-1) or murine B7-2/hygromycin (CHO-Ad/B7-2) and maintained in DMEM supplemented with fetal calf serum and appropriate selection media (*23*). The parent CHO/I-A^d cell line was a gift of Dr. Andrea Sant (University of Chicago) and the murine B7-2/hygromycin construct was provided by Dr. Gordon Freeman (*26*).

Diabetes. Diabetes was induced in 7–10-week-old B6 mice by streptozotocin injection (175 mg/kg body weight). Nonfasting blood glucose levels were determined on blood obtained from the tail vein. Only mice with nonfasting blood glucose levels of over 300 mg/dl were used as islet transplant recipients.

Mouse islet isolation and transplantation. C3H/HeN mouse islets of Langerhans were separated by collagenase digestion using a modified automated method for human islet isolation (*33, 34*). Briefly, the pancreatic duct of the mouse was cannulated, and 2 ml of Hanks' solution containing 1 mg/ml collagenase (type P, lot 32, Boehringer-Mannheim, Indianapolis, IN) was injected. Thirty-five distended pancreases were then placed in a digestion chamber and subjected to 12 min of enzymatic digestion. The separated islets were purified on a discontinuous Euro-Collins-Ficoll gradient (Sigma, St. Louis, MO). (Densities were 1.108, 1.096, 1.069, and 1.037.) The purified islets were cultured overnight in medium (RPMI 1640 [GIBCO, Grand Island, NY] plus 10% FCS [Hyclone, Logan, UT], 1% penicillin, and streptomycin [Sigma]) at 37°C in 5% CO_2. The following morning, 500 C3H islets were transplanted beneath the left renal capsule of diabetic B6 mice in the absence or presence of the immunosuppressive agent (50 μg of each antibody every other day for 14 days beginning on the day of transplantation). Animals receiving a combination of anti-B7-1/anti-B7-2 mAbs received 50 μg of each mAb. The nonfasting blood glucose levels were determined in all animals prior to and daily after transplantation. Rejection of the islet allografts was considered to have occurred when nonfasting blood glucose concentrations exceeded 350 mg/dl for 3 consecutive days. Histological examination of the islet grafts was performed on selected animals to confirm graft rejection.

Histological studies. Renal/transplant histology was performed on tissue sections stained with hematoxylin-eosin after paraffin embedding to identify the inoculated islets and assess the extent of rejection. Nephrectomy specimens were fixed in formalin, paraffin embedded, and sectioned at 5 microns for routine histology and immunohistochemical analysis. Staining for insulin utilized a primary goat antiserum to insulin (DAKO, Carpenteria, CA) followed by the avidin-biotin peroxidase method, and diaminobenzidine as substrate. Nonimmune guinea pig serum and a normal human pancreas served as negative and positive controls, respectively. Immunohistochemical staining for CD4 and CD8 cell surface expression was performed as described previously (*35*). Briefly, nephrectomies were performed on kidneys containing the islet graft at 10 days posttransplant. The fresh tissues were snap-frozen, and 6-μm sections were fixed in Zamboni's fixative for 1 hr, then stained with the individual mAbs: anti-CD4 (GK1.5) and anti-CD8 (3.168) (gifts from Dr. Frank Fitch, University of Chicago) or an irrelevant rat control mAb (PharMingen, San Diego, CA): affinity-purified, mouse absorbed rabbit antirat Ig-biotin and avidin-horseradish peroxidase conjugates (Vector, Burlingame, CA). Background peroxidase activity was reduced by incubating sections in 0.1% azide after staining with rabbit antirat Ig biotin secondary antibodies. Immunohistochemical slides were scored blindly for the number of $CD4^+$ and $CD8^+$ T cells infiltrating the islet grafts. An arbitrary unit (AU) was defined as the number of positive cells/field. At least two areas of each transplant were scored and then averaged. The scores are representative of the mean of three mice per treatment group.

T cell proliferation assays. Transfectant assay: CHO cell transfectants were mitomycin-C (Sigma)–treated for 90 min, washed extensively, and then pulsed with 25 μg/ml of tryptic digest of OVA (DOT). PGL10 (Th1 clone) at 5×10^4 cell/well was incubated with 5×10^4 antigen-pulsed CHO transfectants/well in the presence of 10 μg/ml of the indicated blocking antibodies. The assays were pulsed with ^{3}H-thymidine at 48 hr and harvested 16 hr later. Percent inhibition is in reference to cultures without blocking antibodies.

Allogeneic MLR/MLIC: spleen cells were plated at 5×10^6 cells per well (24-well plate) in 1 ml of complete medium containing either lipopolysaccharide (LPS) (10 μg/ml) or anti-Ig (10 μg/ml) for 48–72 hr. Then 2.5×10^5 irradiated splenocytes (2000 rads), or activated B cells (1200 rads), or 50 irradiated islet cells (2000 rads) used as stimulators were treated with blocking or control antibodies for 15 min. Purified B6 T cells, prepared by pretreatment of isolated lymph node cells with a cocktail of anti-HSA (J11D) (*36*) and anti-I-A^b (25-9-3, ATCC) mAbs and complement (Pelfreez), were added at a density of $1–5\times10^5$ cells/well as stated. The 96-well plates were incubated at 37°C for 72–96 hr, pulsed with 1 μCi [^{3}H]-thymidine/well, and harvested 12 to 16 hr later. T cell purity was confirmed by flow cytometric analyses and was greater than 98% pure (data not shown). Counts are represented as mean CPM of triplicate wells. SEM were < 20%.

Cytokine assays. Cytokines were detected in culture supernatents by ELISA using commercially available kits for IL-2 and IL-4 (Endogen, Cambridge, MA), and reagents for IFN-gamma were kindly provided by Dr. Bob Schrieber (Washington University St. Louis, MO).

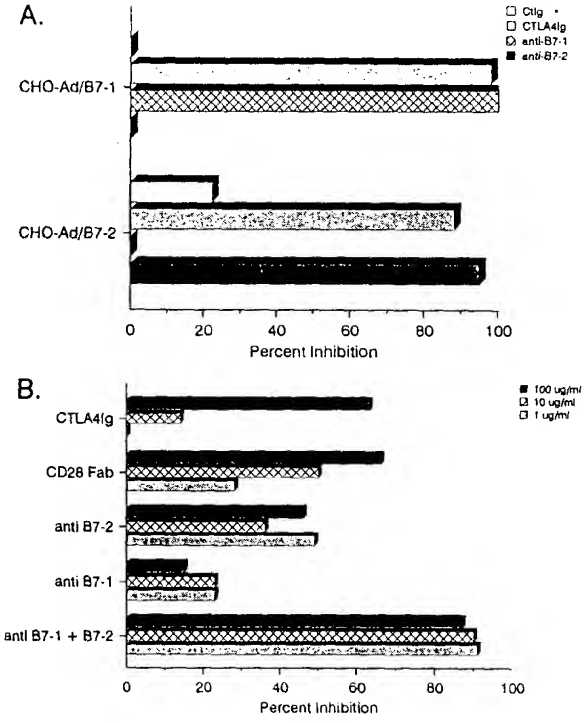

FIGURE 1. Inhibition of a primary allogeneic MLR by CD28 antagonists. (Panel A) A Th1 clone (PGL10) was incubated with mitomycin-treated CHO cells transfected with I-A^d and either murine B7-1 or B7-2 that had been pulsed with tryptic digest of OVA (DOT). CHO cells expressing only I-A^d, B7-1, or B7-2 induced no proliferation. The assay was carried out in the presence of 10 μg/ml of the indicated antibodies. These data demonstrate that the anti-B7-1 and anti-B7-2 mAbs do not crossreact on each other and that they are capable of inhibiting a B7-1- and B7-2-induced response, respectively. (Panel B) C57BL/6 lymph node cells were stimulated with C3H/HeN splenocytes in the presence or absence of the CD28 antagonists. Although the inhibitory effects of the CTLA4Ig and anti-CD28 Fab fragments decreased following titration of the reagents, the anti-B7-1 and anti-B7-2 mAbs inhibited maximally, even at the lowest concentrations. Data in both panels are represented as percent inhibition of the response in the absence of any antibodies and are representative of two experiments.

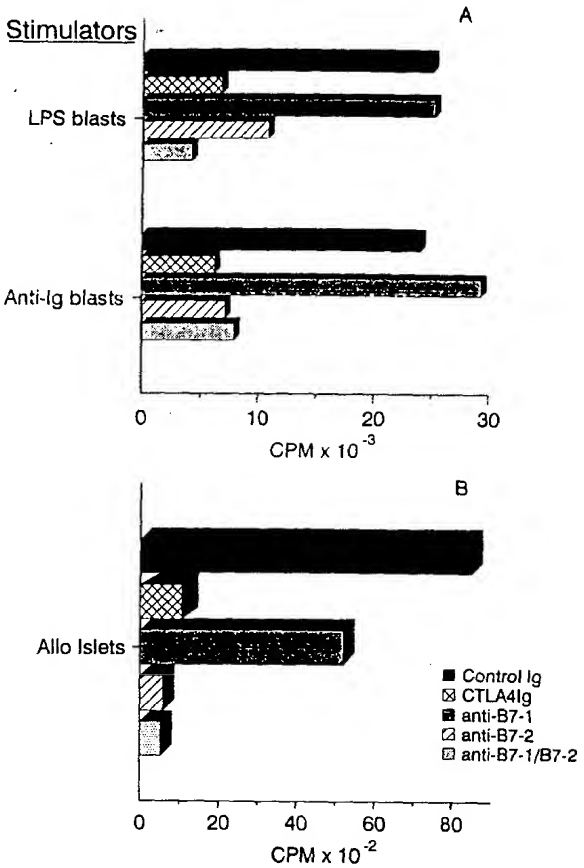

FIGURE 2. Anti-B7 antibodies differentially inhibit T cell proliferative responses using different APCs. Purified B6 T cells were incubated with either irradiated C3H/HeN B cell blasts that had been activated by LPS or anti-Ig (panel A) or with 50–100 C3H/HeN islets (panel B). No proliferation was observed using syngeneic B cells, and an antihamster control antibody did not inhibit proliferation (data not shown). Results are representative of three experiments.

RESULTS

The role of B7-1 and B7-2 cell surface molecules in in vitro costimulation of T cell proliferation. Initial studies were performed to determine the costimulatory activity of B7-1 and B7-2 in vitro during an allogeneic MLR and MILR. First, we compared the ability of CTLA4Ig (a CD28 antagonist that binds both B7-1 and B7-2), anti-B7-1, and anti-B7-2 mAbs to inhibit T cell proliferation to B7-1 or B7-2 transfectants with allogeneic splenic APCs. As seen in Figure 1A, both the anti-B7-1 and anti-B7-2 mAbs had equivalent functional activity when used to block the antigen-specific proliferation of a Th1 clone (PGL10) to the appropriate transfectants. For instance, anti-B7-1 mAb (16-10A1) effectively blocked the proliferative response of PGL10 to CHO cell transfectants expressing I-A^d and B7-1 but not B7-2. By comparison, anti-B7-2 mAb (GL-1) inhibited the proliferative response of PGL10 to CHO-I-A^d cells cotransfected with B7-2 but not

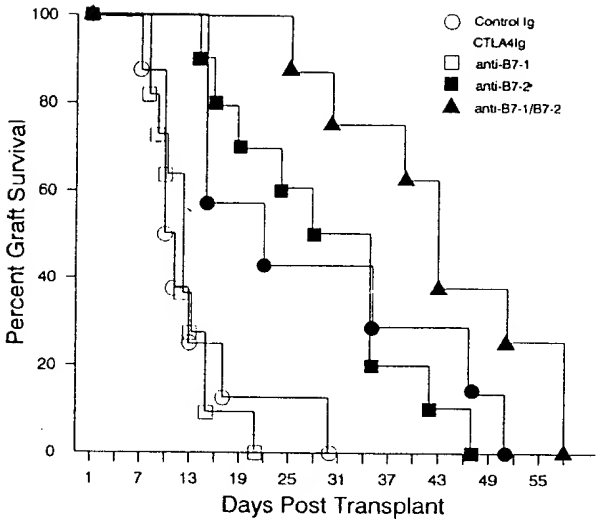

FIGURE 3. Survival of murine pancreatic islet allografts. Purified C3H/HeN pancreatic islet cells were transplanted into diabetic C57BL/6 mice (nonfasting glucose levels >300 mg/dl). Mice were injected, i.p., every other day (50 μg/mouse) for 14 days with the various blocking antibodies, and graft survival was monitored by following daily blood glucose levels. Graft survival time of animals treated with control Ig (open circles, 13.5 days, $n=8$), CTLA4Ig (closed circles, 28.6 days, $n=7$), anti-B7-1 (open squares, 12.3 days, $n=11$), anti-B7-2 (closed squares, 29.5 days, $n=10$), as well as combined anti-B7-1/anti-B7-2–treated islet grafts (closed triangles, 43.4 days, $n=8$) were compared. There was no statistically significant difference between the survival time of the anti-B7-1- and the control Ig–treated groups or between the CTLA4Ig and anti-B7-2-treated groups. However, CTLA4Ig, anti-B7-2, and combined antibody treatment demonstrated statistically prolonged allograft survival as compared with the control Ig– and anti-B7-1-treated groups ($P=<0.001$).

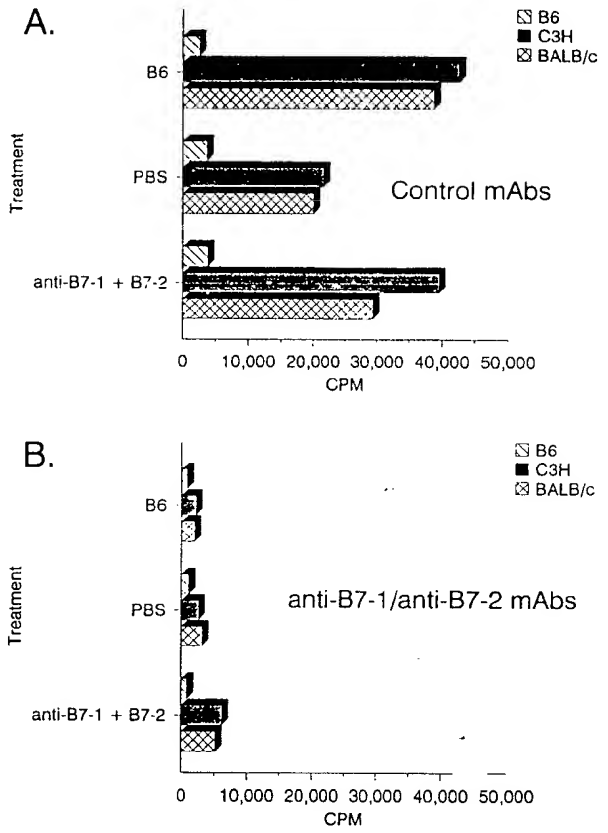

FIGURE 4. In vitro function of T cells isolated from anti-B7-1/anti-B7-2-treated mice. (Panel A) Splenic T cells were isolated from treated mice at either 10 (data not shown) or 20 days posttransplant and stimulated with donor (C3H/HeN, shaded bar) or third-party (BALB/c, crosshatched bar) spleen cells as described in *Materials and Methods*. At both time points there was no difference between the PBS-treated and anti-B7-1-/B7-2-treated mice in response to either donor or 3rd-party stimulators. (Panel B) The same cultures were set up in the presence of a combination of anti-B7-1 and anti-B7-2 mAbs (10 μg/ml of each mAb). Proliferation in these cultures was inhibited, while control antibodies had no effect. The data represent the mean of triplicate cultures and are representative of two experiments.

B7-1. Human CTLA4Ig and anti-CD28 Fab fragments (data not shown) blocked the proliferative responses to both transfectants. Similar findings were observed when the CHO cell transfectants were used to stimulate an allogeneic response with purified T cells (data not shown). In contrast, T cell proliferation to allogeneic splenic APCs was only inhibited by anti-B7-2, but not anti-B7-1 mAbs (Fig. 1B). In repeated experiments, the anti-B7-2 mAb inhibited proliferative responses between 50 and 75% while the anti-B7-1 mAbs only marginally inhibited (<15% in most experiments). Moreover, hCTLA4Ig inhibited the proliferative response of purified T cells to allogeneic spleen to a greater extent than either antibody alone. Inasmuch as CTLA4Ig appears to react exclusively with B7-1 and B7-2 on these APCs—as a combination of the two antibodies block CTLA4Ig binding to these stimulator cells, (*29*)—the anti-B7-1 and anti-B7-2 mAbs were added simultaneously to the cultures. In repeated experiments, the combination of the anti-B7-1 and anti-B7-2 mAbs inhibited the T cell proliferative response to a greater degree than the anti-B7-2 alone (Fig. 1B). In fact, the combined anti-B7-1 and anti-B7-2 mAbs inhibited the MLR better than CTLA4Ig, reflecting the increased affinity of these mAb reagents for their respective ligands. Interestingly, the combined antibodies inhibited the MLR at doses as low as 1 μg/ml. These doses are far less than routinely observed for blocking mAbs such as anti-CD28 Fabs, CTLA4Ig or anti-CD4 and anti-CD8 mAbs.

Previous studies have shown that B7-1 and B7-2 are differentially expressed on distinct APC populations (*29*, *23*). For instance, dendritic cells, which are the primary splenic stimulator cells of an allogeneic MLR (*37*) express both B7-1 and B7-2, while anti-Ig-stimulated B cell blasts preferentially express B7-2. Therefore, we compared the contribution of B7-1 vs B7-2 on distinct APCs, including LPS or anti-Ig–stimulated B cell blasts and purified islet cells (Fig. 2). While all of the responses were significantly inhibited by anti-B7-2 mAbs, there were differences in the role played by B7-1. The T cell proliferative response to splenic LPS blast cells was dependent on both B7-1 and B7-2 since the combination of the two mAbs inhibited the response more than either alone.

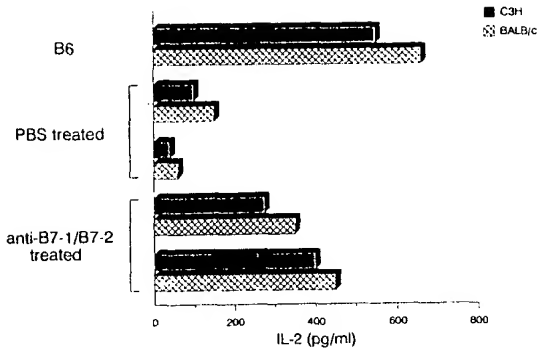

FIGURE 5. IL-2 production by T cells from transplanted mice in response to donor vs. 3rd-party stimulators. T cells from untreated B6 mice or from islet grafted mice were isolated 10 days posttransplant and cultured with either donor (C3H) or 3rd-party (BALB/c) stimulators as described in *Materials and Methods*. Supernatants were collected at 48 hr and assayed for IL-2, IL-4, and gamma interferon by ELISA. No detectable levels of IL-4 were observed in these cultures. Low levels of gamma interferon were observed, but there were no differences in the anti-B7-1/B7-2-treated mice in response to donor versus 3rd-party APCs (data not shown).

In contrast, only anti-B7-2 mAb inhibited the T cell proliferative response to alloantigen expressed on anti-Ig B blasts (panel A). These findings are consistent with previous studies demonstrating that B cells stimulated by anti-Ig expressed B7-2 but not B7-1, while LPS-stimulated B cells expressed both (*29*). Like the allogeneic spleen cells, the proliferative response of T cells stimulated by the allogeneic islet cells was significantly blocked by the B7-2-specific mAb and, to some degree, by the B7-1-specific mAbs (panel B). In all instances, CTLA4Ig blocked the allorecognition. Together, these results suggest that B7-2 is a major costimulatory molecule of an allogeneic MLR and MILR in vitro, but that B7-1 may also play an important costimulatory role depending on the source of the antigen-presenting cells. Finally, even under optimal conditions, the combined antibody treatment did not fully inhibit the allogeneic MLR. Thus, there are likely to be additional molecules, either soluble or cell surface–bound, that can costimulate an allogeneic response. These additional costimulator pathways may involve cytokines such as IL-1 or contact-mediated costimulatory molecules such as CD43 or ligands present on monocytes (*38, 39*).

CTLA4Ig and anti-B7-2 mAb, but not anti-B7-1 mAb, block allogeneic pancreatic islet graft rejection. The in vitro analyses of the mAbs suggested that B7-2 is a primary costimulator of responses to allogeneic islet cells. In order to determine the relative role of B7-1 and B7-2 in vivo, B6 mice were transplanted with 500 purified allogeneic (C3H/HeN) islets under the left kidney capsule in the presence or absence of B7-1 and B7-2 antagonists. Mice were treated with 50 μg of hCTLA4Ig, anti-B7-1 mAb, anti-B7-2 mAb, or 50 μg of both anti-B7-1 and anti-B7-2 mAbs every other day for 14 days. Therapy with hCTLA4Ig, which blocks both B7-1 and B7-2 costimulation, inhibited allograft rejection in this model (Fig. 3), consistent with previous studies by our laboratory and others demonstrating that CTLA4Ig treatment suppressed xenograft and allograft rejection. Next, the ability of anti-B7-1 or anti-B7-2 mAbs to block the allogeneic graft rejection was examined. Anti-B7-1 mAbs had no significant effect on graft survival—however, anti-B7-2 mAb therapy was as efficacious as hCTLA4Ig in blocking graft rejection (Fig. 3). These results, combined with the in vitro studies, suggest that B7-2 plays a principal role in primary allogeneic responses to islet cells in vivo and suggest that the initial antagonistic activity of CTLA4Ig is the blockade of B7-2 ligation. Interestingly, in vivo therapy using a combination of anti-B7-1 plus anti-B7-2 mAbs (Fig. 3) significantly prolonged the mean survival time of the grafts beyond either the CTLA4Ig or anti-B7-2 alone. Although the present studies

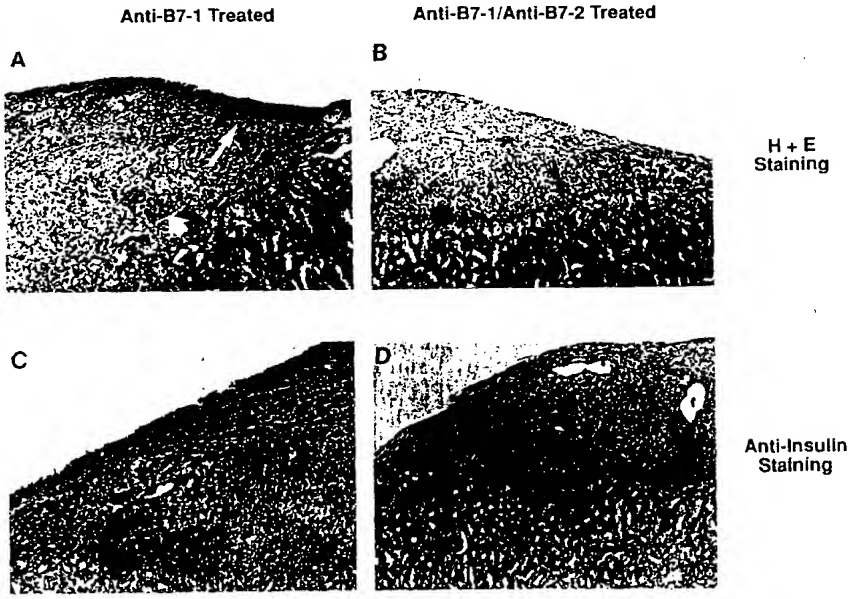

FIGURE 6. Histological examination of islet tissue grafted under the renal capsule of B6 mice. (Panels A and B) A representative section of an islet transplant from a B6 mouse recipient 10 days after allotransplant and treatment with either anti-B7-1 (panel A) or anti-B7-1/anti-B7-2 (panel B). A massive lymphocytic infiltrate (thin white and black arrows) was observed. However, intact islets (thick white and black arrows) were also noted. (H & E, original magnification, ×20). (Panels C and D) Insulin-specific staining of transplanted islets from the same recipients as in (A and B) shows insulin granules present in both sections (×120).

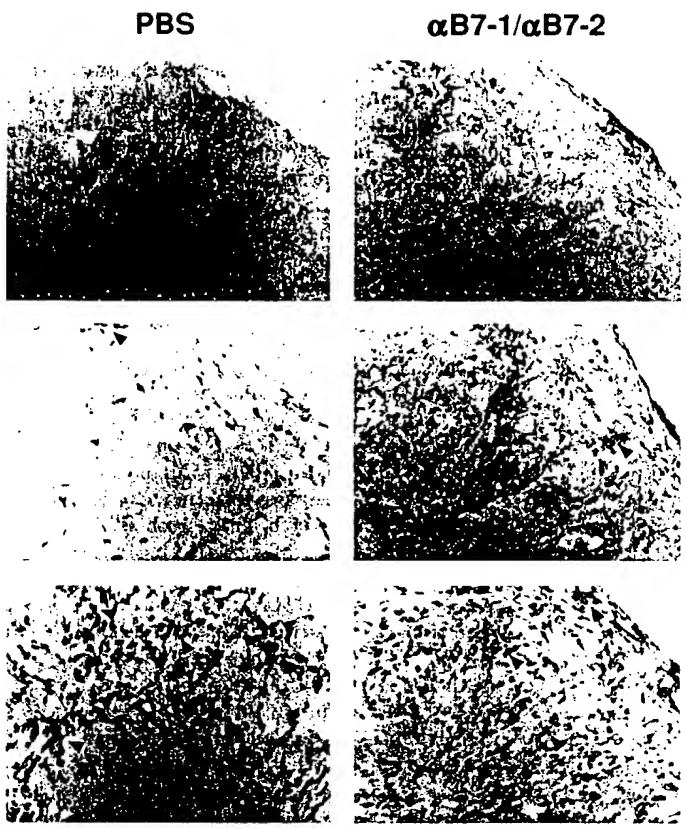

FIGURE 7. Immunohistochemical staining of islet tissue from PBS and anti-B7-1/anti-B7-2-treated mice. Frozen sections of islet transplant specimens from B6 recipient mice 10 days after allotransplantation were stained with control Ig, anti-CD4, or anti-CD8 mAbs (original magnification, ×120). Both $CD4^+$ and $CD8^+$ T cells (black arrows) were observed in both the PBS and anti-B7-1/anti-B7-2–treated islet grafts. However the number of $CD8^+$ T cells (PBS=840 AU; anti-B7-1/anti-B7-2=902 AU) was significantly greater than the number of $CD4^+$ T (PBS=250 AU; anti-B7-1/anti-B7-2=113 AU) cells infiltrating into the grafts. Furthermore in the anti-B7-1/anti-B7-2–treated mice there was a decreased number of $CD4^+$ T cells (113 AU) as compared with PBS-treated mice (250 AU). An arbitrary unit (AU) was defined as the relative number of positive cells/field, with at least two areas of each transplant being scored and then averaged.

cannot distinguish between selective effects of the two costimulatory molecules on the same APCs or selective expression of B7-1 and B7-2 on different APCs, it is clear that B7-1 plays a secondary but substantive role in allogeneic responses to islet cells in vivo.

Functional and histological analyses of CD28 antagonist–treated mice. Next, we examined the mechanism by which the CD28 antagonists prolonged graft survival. T cell responses from treated animals were examined at various time points posttransplantation. At either 10 days (data not shown) or 20 days posttransplant (Fig. 4A) both PBS- and anti-B7-1/anti-B7-2–treated mice responded to donor or 3rd-party stimulators. The anti-B7-1/anti-B7-2–treated mice, which were still maintaining their allogeneic islet grafts, proliferated equivalently to C3H (donor) and BALB/c (3rd-party) splenic APCs (Fig. 4A). Even at 10 days posttransplant, while the animals were still being treated, the responses were equivalent (data not shown). Furthermore, the anti-B7-1/anti-B7-2–treated mice made equivalent amounts of IL-2 (Fig. 5) and gamma interferon (data not shown) in response to either stimulator. However, no IL-4 could be detected at any time in these treatment groups. Finally, this proliferative response was still dependent upon the CD28/B7 costimulation pathway, since a combination of anti-B7-1 and anti-B7-2 mAbs inhibited this response in both the PBS and anti-B7-1/anti-B7-2–treated mice (Fig. 4B). Therefore, T cells isolated from immunosuppressed mice respond normally to in vitro challenge with allogeneic antigen by utilizing a B7-1/B7-2-dependent signaling pathway.

Next, we examined the different treatment groups for any differences in the makeup of the infiltrate into the transplant site. At 10 days posttransplant a severe lymphocytic infiltrate was observed in all treatment groups, and only marginal differences in insulin secretion were observed (Fig. 6). Because there were no systemic differences in T cell function in the immunosuppressed mice, we performed immunohistochemical staining to examine lymphocyte subsets entering the allografts. As seen in Figure 7, in both treatment groups the majority of T cells infiltrating the islet graft were $CD8^+$ T cells (PBS relative number=840 AU; anti-B7-1/anti-B7-2 relative number=902 AU). $CD4^+$ T cells were also present in both groups. However, the relative number of $CD4^+$ T cells was significantly decreased in the anti-B7-1/anti-B7-2–treated animals as compared with the PBS treated animals (PBS=250 AU; anti-B7-1/anti-B7-2=113 AU), suggesting that while this therapy did not prevent lymphocytic infiltrate, it did alter the infiltration of $CD4^+$ T cells into the grafts. Interestingly, other mAb therapies, such as anti-ICAM-1, completely block all leukocytic infiltration that follows graft transplantation by blocking the leukocyte interaction with the endothelium and preventing the development of the initial inflammatory response and cytokine release (*40*). In contrast, the anti-B7 mAbs appear to block a later stage in

the immune reaction—namely, the sensitization of the antigen-specific T cells following APC presentation either in the graft or draining lymph node cells.

DISCUSSION

The present study illustrates the importance of the CD28 costimulation pathways, especially CD28/B7-2 interactions, in allogeneic transplant responses. While anti-B7-1 mAb therapy had little effect on islet graft prolongation, human CTLA4Ig, anti-B7-2 mAbs, and a combination of anti-B7-1/anti-B7-2 mAb therapy significantly prolonged islet graft survival. These results differ from recent findings in which anti-B7-1 mAb altered the immune response. In one case, the treatment of mice during the initiation of EAE with anti-B7-1 mAb protected mice from disease onset while anti-B7-2 mAbs exacerbated disease (*31*). In another study, anti-B7-1 mAb treatment of NOD mice prior to insulitis resulted in an accelerated disease onset, while anti-B7-2 treatment prevented diabetes (*30*). Together, these results suggest that depending upon the status of the immune response and the APCs being utilized in the response, the roles of B7-1 and B7-2 may vary. We also examined the mechanism of graft prolongation induced by anti-B7-1/anti-B7-2 treatment. We were unable to identify significant alterations in T cell function in immunosuppressed transplanted mice, although $CD4^+$ T cell infiltration was significantly decreased in the anti-B7-1/anti-B7-2–treated mice. These observations mimic results previously observed in the xenogeneic human islet transplant model. Despite evidence of donor-specific graft tolerance in vivo in the xenogeneic model, no immune dysfunction has been observed in vitro (*19*, and Lenschow DJ, et al. unpublished results). Current studies are underway to determine if regulatory cells have been induced or if the relevant antigen-specific T cells have been diverted from the lymphoid tissues, resulting in a diminished alloreactive response in vitro.

Finally, the CTLA4Ig or combined anti-B7-1/anti-B7-2 therapies did not result in permanent donor-specific allograft tolerance. These results are distinct from those previously observed using a xenogeneic human islet transplant model. The dissimilarity between the two systems may be a result of several, not mutually exclusive, differences between allogeneic and xenogeneic reactions, including a distinct role of host vs. donor APCs (direct presentation of antigen from donor APCs or an indirect presentation pathway wherein foreign antigens are shed and represented on host APCs) in the two transplant systems; the presence of redundant costimulatory pathways operating in the allogeneic reaction; or a distinct difference in the strength of the response. Interestingly, it has been recently reported that either a combination of hCTLA4Ig plus a donor-specific transfusion or delaying treatment with CTLA4Ig until two days posttransplant led to permanent tolerance in two different rat allograft models (*20*, *22*). Thus, it is possible that a strong TCR signal (signal one; provided by the donor cells or the xenogeneic islets) is necessary to maximize anergy induction following B7-2 blockade. Further studies will be needed to clarify the differences in the distinct systems before these therapies can be exploited optimally in the clinical setting.

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Appendix G

Differential Effects of Anti-B7-1 and Anti-B7-2 Monoclonal Antibody Treatment on the Development of Diabetes in the Nonobese Diabetic Mouse

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Summary

Insulin-dependent diabetes mellitus (IDDM) is thought to be an immunologically mediated disease resulting in the complete destruction of the insulin-producing islets of Langerhans. It has become increasingly clear that autoreactive T cells play a major role in the development and progression of this disease. In this study, we examined the role of the CD28/B7 costimulation pathway in the development and progression of autoimmune diabetes in the nonobese diabetic (NOD) mouse model. Female NOD mice treated at the onset of insulitis (2–4 wk of age) with CTLA4Ig immunoglobulin (Ig) (a soluble CD28 antagonist) or a monoclonal antibody (mAb) specific for B7-2 (a CD28 ligand) did not develop diabetes. However, neither of these treatments altered the disease process when administered late, at >10 wk of age. Histological examination of islets from the various treatment groups showed that while CTLA4Ig and anti-B7-2 mAb treatment blocked the development of diabetes, these reagents had little effect on the development or severity of insulitis. Together these results suggest that blockade of costimulatory signals by CTLA4Ig or anti-B7-2 acts early in disease development, after insulitis but before the onset of frank diabetes. NOD mice were also treated with mAbs to another CD28 ligand, B7-1. In contrast to the previous results, the anti-B7-1 treatment significantly accelerated the development of disease in female mice and, most interestingly, induced diabetes in normally resistant male mice. A combination of anti-B7-1 and anti-B7-2 mAbs also resulted in an accelerated onset of diabetes, similar to that observed with anti-B7-1 mAb treatment alone, suggesting that anti-B7-1 mAb's effect was dominant. Furthermore, treatment with anti-B7-1 mAbs resulted in a more rapid and severe infiltrate. Finally, T cells isolated from the pancreases of these anti-B7-1–treated animals exhibited a more activated phenotype than T cells isolated from any of the other treatment groups. These studies demonstrate that costimulatory signals play an important role in the autoimmune process, and that different members of the B7 family have distinct regulatory functions during the development of autoimmune diabetes.

Diabetes is an autoimmune disease that results in the destruction of the insulin-producing islet cells (1). Despite the development of new tools for the identification of individuals who are at risk for developing insulin-dependent diabetes mellitus (IDDM),[1] there are limited therapeutic options to offer these future patients. Thus, current attempts to develop useful immunosuppressive therapies depends on a more complete understanding of the pathogenesis of disease.

Illness in the nonobese diabetic (NOD) mice shares many common features with human IDDM, and this mouse strain has provided an important model for dissecting the pathogenesis of autoimmune diabetes (2, 3). NOD mice spontaneously develop insulitis early in life (between 2 and 4 wk of age). However, it is not until 10–20 wk later that this insulitis progresses to diabetes in ~80% of the female mice and in only 20% of the male mice. As in human IDDM, there is extensive evidence supporting a role for T cells and

[1] *Abbreviations used in this paper:* CtIg, control Ig; GAD, glutamic acid decarboxylase; H&E, hematoxylin and eosin; IDDM, insulin-dependent diabetes mellitus; MFI, mean fluorescence intensity; NOD, nonobese diabetic.

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MHC-restricted self-antigen recognition in the development of disease. First, T cells are among the earliest infiltrating cells in diseased islets (4, 5). Treatment of NOD mice with antibodies directed at T cell surface molecules such as CD4, CD8, and the TCR/CD3 complex prevent the development of disease and, in the case of anti-CD3 mAbs, blocked the progression of diabetes in a diabetic animal (6–8). Furthermore, diabetes can be precipitated by adoptively transferring an islet-specific clone into young NOD mice (9, 10).

One hypothesis for the development of autoimmune disease is that the disruption of the normal mechanisms of peripheral tolerance may occur. For instance, recent studies have shown that the development of insulitis and diabetes in the NOD mouse correlates with the acquisition of T cell reactivity to glutamic acid decarboxylase (GAD) and a series of other islet antigens (11, 12). Indeed, NOD mice rendered tolerant to GAD by intravenous or intrathymic injection showed a lower incidence of IDDM compared with control animals. Unfortunately, the therapeutic potential for tolerizing islet-specific T cells before their encounter with antigen is limited. Therefore, efforts have been devoted to altering the functional activity of autoreactive T cells after antigen recognition in an attempt to promote a tolerant rather than an activated state. Recent work has demonstrated that in addition to TCR engagement by antigen, a second signal, known as costimulation, is also required for T cell activation (13, 14). Blockade of this costimulatory signal results in the induction of a state of antigen-specific nonresponsiveness known as anergy (13). Thus, one potential approach to inducing autoreactive T cells into a tolerant state might be to block these costimulatory events. Studies performed in transgenic mice support this possibility. The ecotopic expression of MHC class I or II molecules on nonprofessional APCs, such as the islets of Langerhans, is not enough to activate potentially autoreactive T cells (15, 16). However, the coexpression of the appropriate costimulatory molecules, such as B7-1, on the islets activates these cells and results in the autoimmune destruction of the islets (17–19). Therefore, the potential to regulate T cell costimulation provides a potent new approach to altering the functional activity of autoreactive T cells.

While the costimulatory signals involved in IDDM are currently unknown, there are many cell surface molecules that may deliver the necessary costimulatory signal. Evidence suggests that the major T cell costimulatory pathway involves the CD28-B7 family of costimulatory molecules. CD28 is expressed on the majority of naive and memory T cells (20, 21). Activation of T cells with anti-CD28 mAb blocks the induction of anergy and synergizes with anti-CD3 stimulation to increase both T cell proliferation and lymphokine production in vitro (22–25). Furthermore, F(ab) fragments of anti-CD28 inhibit the activation of T cells and, in some instances, renders them anergic (22).

There are two known natural ligands for CD28. B7-1 was the first ligand to be identified and is expressed on "professional" APC, such as dendritic cells, macrophages, and activated B cells (26–30). In vitro studies using B7-1 transfectants demonstrated that B7-1 costimulated both antigen- and mitogen-driven T cell proliferation and IL-2 production by interacting with CD28 (31, 32). Recently, we and others have identified a second CD28 ligand, B7-2, which is also expressed on "professional" APC (33–36). Cloning of the B7-2 molecules has revealed sequence similarity to B7-1, and it has been shown to bind to the CD28 molecule (37–39). Antibodies to B7-2 are more effective than anti-B7-1 mAb at blocking T cell responses to natural APC, such as in an allogeneic mixed lymphocyte reaction (40, 41). Furthermore, prolongation of allogeneic islet graft survival occurred under the cover of anti-B7-2 but not anti-B7-1 mAb treatment, suggesting that the B7-2 costimulatory molecule plays a more dominant role than B7-1 in this immune response (Zeng, J., Lenschow, D. J., and Bluestone, J. A., manuscript submitted for publication).

CTLA-4 is a cell surface molecule, with sequence homology to CD28, expressed on activated $CD4^+$ and $CD8^+$ T cells (42, 43). Although CTLA-4 may not function as a costimulatory molecule, a soluble fusion protein comprising the extracellular domain of CTLA-4 and the Fc portion of human IgG1 constant region, CTLA4Ig, binds both B7-1 and B7-2 and has been used to inhibit a variety of CD28-dependent immune responses, including in vivo antibody responses and allogeneic and xenogeneic graft rejection (44–46). In fact, in a xenogeneic islet transplant model, blockade of the CD28/B7 costimulatory pathway with CTLA4Ig led to the induction of donor-specific tolerance (46).

In this study, we examined the role of the CD28/B7 signaling pathway in the initiation and propagation of autoimmune diabetes in the NOD mouse. While blockade of this costimulatory pathway with either CTLA4Ig or anti-B7-2 mAb prevented disease development, treatment with anti-B7-1 or a combination of anti-B7-1 and anti-B7-2 mAbs resulted in a more rapid onset of disease in both female and male mice. These results indicate that the CD28/B7 costimulatory pathway is involved in the control of this autoimmune response and may provide a powerful target to alter the function of autoreactive T cells and human disease progression.

Materials and Methods

Mice. NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms, Inc. (Germantown, NY) and were bred in a specific pathogen–free animal facility at the University of Chicago. C57BL/6 (B6) mice were purchased from The Jackson Laboratory and were maintained in a pathogen-free animal facility at the University of Chicago.

Antibodies. In initial studies, human CTLA4Ig and a control fusion protein, L6Ig, were provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA) (47). Later studies were performed with CTLA4Ig provided by Repligen Corp. (Cambridge, MA) (33) and a control human Ig purchased from Sigma Chemical Co. (St. Louis, MO). Similar results were obtained with both sets of reagents. The hamster anti–murine B7-1 mAb (16-10A1) was provided by Repligen Corp. The rat anti–murine B7-1 mAb (1G10) was generated as previously described (48). The rat anti–murine B7-2 mAb (GL1) was produced in an Acusyst Jr. bioreactor (Coons

River, MN) and was purified by passage over a protein G–coupled Sepharose column (35). FITC-coupled anti-Thy1 and anti-B220 mAbs and biotin-coupled anti-CD4, anti-CD8, and anti-CD69 were purchased from PharMingen (San Diego, CA). PE-coupled streptavidin was purchased from Southern Biotechnology Associates (Birmingham, AL).

Treatment Protocol. Groups of NOD mice were treated with CTLA4Ig, anti-B7-1 mAbs (16-10A1 and 1G10), anti-B7-2 mAb (GL1), and control Abs (L6Ig and CtIg) as follows. Male and female NOD mice between 2 and 3 wk of age were treated with 50 μg of either control Abs (L6Ig or CtIg), CTLA4Ig, anti-B7-1 mAb (1G10 or 16-10A1), or anti-B7-2 mAb (GL1) every other day for 14 d. Animals then received one additional 50-μg dose at weeks 6, 7, and 8. Animals receiving both anti-B7-1 (16-10A1) and anti-B7-2 mAb treatment received 50 μg of each mAb. To test the effects of these reagents on later stages of the disease, female NOD mice were treated with 50 μg of the above reagents three times per week starting at 80 d of age for ~10 wk, or until the animals became diabetic. Animals treated with control antibodies beginning at 2 wk of age showed a slight delay in the development of diabetes (12 wk) compared with untreated mice (10 wk) although the penetrance of disease was the same by 24 wk of age, with nearly 90% of both control treated and untreated female mice becoming diabetic by 24 wk of age.

Assessment of Diabetes. Starting at 7 wk of age, diabetes was assessed by weekly measurements of blood glucose levels by use of a glucose meter (One Touch II; Lifescan, Inc., Milpitas, CA). Glucose strips were kindly provided by Lifescan, Inc. Animals were considered diabetic after two consecutive measurements >250 mg/dl. Onset of diabetes was dated from the first of the sequential diabetic measurements.

Histological Analysis. The pancreases from killed animals were fixed in buffered formalin and embedded in paraffin. 4-μm sections were cut and hematoxylin and eosin (H&E) staining was performed. To determine the severity of insulitis in the various treatment groups, four to six animals per time point (4, 8, and 12 wk) were analyzed, and multiple tissue sections of the pancreases for each animal were scored for insulitis. At least 50 islets were counted per time point. Islets were scored blindly and found to be either free of insulitis (score = 0), exhibiting periinsulitis (lymphocytes surrounding the islets and ducts but not infiltrating the islet architecture; score = 1), exhibiting moderate insulitis (lymphocytes infiltrating <50% of the islet architecture; score = 2), or exhibiting severe insulitis (>50% of the islet tissue infiltrated by lymphocytes; score = 3). Mean clinical score = severity score × number islets in that category/number of mice.

Pancreatic Lymphocyte Isolation. Pancreases from animals within the same treatment group were minced into 2–4-mm fragments. These fragments were then digested in an enzyme mixture containing 5 mg of hyaluronidase (Sigma Chemical Co.), 1,500 U of DNase (Sigma Chemical Co.), and 50 mg of collagenase P (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 50 ml of complete media (DMEM, 10% FCS, 2 mM L-glutamine, 25 μM Hepes buffer, 100 U penicillin, 100 μg/ml streptomycin, 2 mM nonessential amino acids, and 5×10^{-5} M 2-ME). This mixture was incubated at room temperature for 60 min with constant stirring, then 1 ml of FCS was added, and the mixture was incubated for an additional 60 min. A single-cell suspension was obtained by filtering through a nytex screen. The cells were washed twice, resuspended in 10 ml media, and then fractionated on a discontinuous BSA density gradient by layering 10 ml of 35% BSA (Sigma Chemical Co.) under 10 ml of 24% BSA, which had been layered under the 10 ml of complete media containing the cells. After centrifugation at 1,400 rpm for 40 min, the interface between the 35 and 24% layers was removed, washed twice, and then analyzed by FACS® (Becton Dickinson & Co., Mountain View, CA) for subsets and activation markers.

Flow Cytometric Analysis. 10^5 spleen cells or pancreatic lymphocytes were washed in FACS® buffer (0.1% BSA and 0.01% sodium azide in 1 × PBS) and then incubated with FITC or biotin-coupled staining reagents for 30 min at 4°C. The cells were then washed in FACS® buffer. Biotin-coupled reagents were developed with PE-coupled streptavidin by staining for 15 min at 4°C. The cells were then washed in FACS® buffer, and two-color flow cytometry was performed by use of a FACScan® flow cytometer (Becton Dickinson & Co.). Data were analyzed with the Lysis II software program (Becton Dickinson & Co.). Data were collected on 10^4 cells based on forward-scatter intensity and exclusion of dead cells based on staining with propidium iodide.

Results

Early Treatment of NOD Mice with CTLA4Ig Prevents the Development of Diabetes. The development of autoimmune disease in the NOD mouse begins at between 2 and 4 wk of age as evident by the infiltration of the islet of Langerhans by lymphocytes (5, 49). This insulitis is an essential step in the development of full-blown diabetes 8–20 wk later. We therefore treated NOD mice beginning at ~2–3 wk of age, presumably at the onset of insulitis. Animals received either CTLA4Ig or control antibody (CtIg) at a dose of 50 μg every other day for 2 wk followed by three additional 50-μg injections at weeks 6, 7, and 8. Female mice were continually monitored for the development of diabetes. While ~87% of the animals treated with CtIg developed disease between weeks 12 and 33, treatment with hCTLA4Ig blocked the development of diabetes (Fig. 1). Only 11% of the treated female mice became diabetic by 33 wk of age in contrast to the 87% of the CtIg-treated animals. Within 24 h after the last injection, we could no longer detect serum levels of CTLA4Ig due to the animals mounting a vigorous antibody response to the human protein (data not shown). These results indicate that a relatively short treatment protocol that interrupts the CD28/B7 costimulatory pathway either by directly signaling or blocking important interactions between members of the CD28/B7 family during a critical time in disease development prevents IDDM in female NOD mice.

CTLA4Ig's Interaction with B7-2 Plays an Important Role in Preventing Disease. CTLA4Ig's ability to inhibit the development of diabetes could have resulted from the binding of CTLA4Ig to either of its two natural ligands, B7-2 or B7-1. We have previously shown that while both molecules have the ability to costimulate T cell responses, under physiologic conditions, B7-2 appears to play the dominant costimulatory role both in vitro and in vivo (33, 40). Anti-B7-2 mAb, but not anti-B7-1 mAbs, inhibits an allogeneic mixed lymphocyte response and prolongs allogeneic islet graft survival (Zeng, J., et al., manuscript submitted for publication). We therefore examined directly the role of B7-2 in the development of autoimmune diabetes in the NOD mouse model.

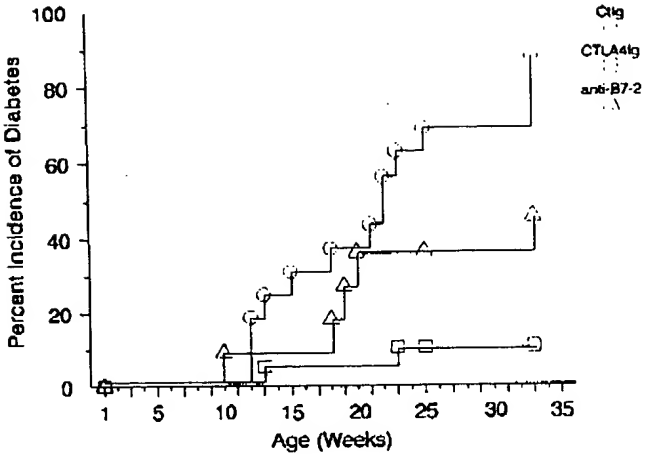

Figure 1. Early treatment of NOD mice with CTLA4Ig and anti-B7-2 mAbs prevents the development of diabetes. Female NOD mice were treated with CTLA4Ig (□; n = 19), anti-B7-2 mAb (△; n = 11), and Ctlg (○; n = 16) as described in Materials and Methods. Beginning at 8 wk of age, diabetes was assessed by weekly measurements of blood glucose levels.

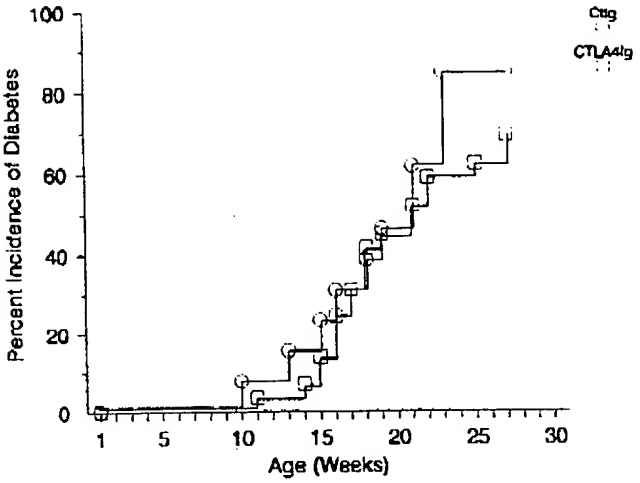

Figure 2. The late treatment of NOD mice has no effect on disease progression. NOD mice were treated with either Ctlg (○; n = 13) or CTLA4Ig (□; n = 29) three times a week for at least 8 wk, or until the development of diabetes, beginning at ~10 wk of age (80 d). The development of diabetes was monitored as described.

2-wk-old NOD mice were treated with the anti-B7-2 mAb (GL1) as described above and followed for the development of diabetes. Only 45% of the anti-B7-2–treated mice became hyperglycemic by 33 wk of age, in contrast to 87% of control treated mice (Fig. 1). Of the five anti-B7-2–treated animals that did develop diabetes, four of them did not develop disease until at least 18 wk of age. Therefore, anti-B7-2 treatment was able to delay disease onset and block disease development in a subset of mice. However, its ability to induce and maintain tolerance to IDDM may not be as efficient as CTLA4Ig, in which only 11% of the treated animals became diabetic.

Late Treatment of NOD Mice with CTLA4Ig Has No Effect on Disease Outcome. While the first evidence of disease can be detected as early as 3–4 wk of age with the occurrence of insulitis, full-blown diabetes does not develop until much later. These observations have suggested that at least two different events must occur to precipitate the eventual development of diabetes. To examine what effect the CTLA4Ig treatment had when initiated well after the induction of insulitis, but before any clinical signs of disease, such as hyperglycemia, had developed, we started treatment of NOD mice when they were 80 d old. Animals were treated three times a week for 10 wk, or until the onset of disease, and monitored for the development of diabetes. Despite the ability of CTLA4Ig to inhibit disease development when administered early, it had little effect on disease outcome when treatment was initiated late in the disease process (Fig. 2). Therefore, it appears that the inhibitory effects of these reagents are exerted during early phases of the disease process, either before the initiation of disease or just subsequent to the initial antigen engagement by islet-reactive cells.

Effects of CD28 Antagonists on the Occurrence of Insulitis. To begin to address the mechanism by which CTLA4Ig and anti-B7-2 prevent disease, we examined treated animals for one of the earliest signs of diabetes, the development of insulitis. Pancreatic tissue from 8-wk-old female mice treated with Ctlg, CTLA4Ig, or anti-B7-2 mAbs were prepared and examined for insulitis. Insulitis could be detected in all of the treatment groups, including the CTLA4Ig and anti-B7-2–treated mice (data not shown). To further examine the development of insulitis in these animals, male and female mice were killed at 4, 8, and 12 wk of age, and histological sections of their pancreases were prepared and scored for the presence of insulitis. Despite the ability of CTLA4Ig and anti-B7-2 mAbs to inhibit the development of disease, the presence of insulitis was readily detectable at all times. By 4 wk of age, ~30% of the islets from Ctlg (mean clinical score = 7.45), CTLA4Ig (mean clinical score = 4.13), and anti-B7-2 mAb– (mean clinical score = 7.25) treated female mice showed evidence of cell infiltration although the majority of the insulitis was nondestructive periinsulitis (Fig. 3). The severity of the infiltrate continued to increase at 8 wk of age in all groups, although both the CTLA4Ig-and anti-B7-2 mAb–treated groups appeared to have a slightly more severe infiltrate at this time than did the Ctlg-treated animals. By 12 wk of age, all three groups had between 40 and 60% of their islets infiltrated by lymphocytes, and the severity had increased (Ctlg mean clinical score = 16.28; CTLA4Ig mean clinical score = 24.0; anti-B7-2 mAb mean clinical score = 16.75). Similar results were also observed in the treated males (data not shown). Thus, the inhibition of disease development induced by CTLA4Ig and anti-B7-2 treatment was not caused by a quantitative difference in the T cell infiltrate into the islets, and therefore these treatments must alter a later event in this disease.

Treatment of NOD Mice with a Combination of Anti-B7-1 and Anti-B7-2 mAbs Accelerates Disease Onset. Previous results in an allogeneic transplant model demonstrated that a com-

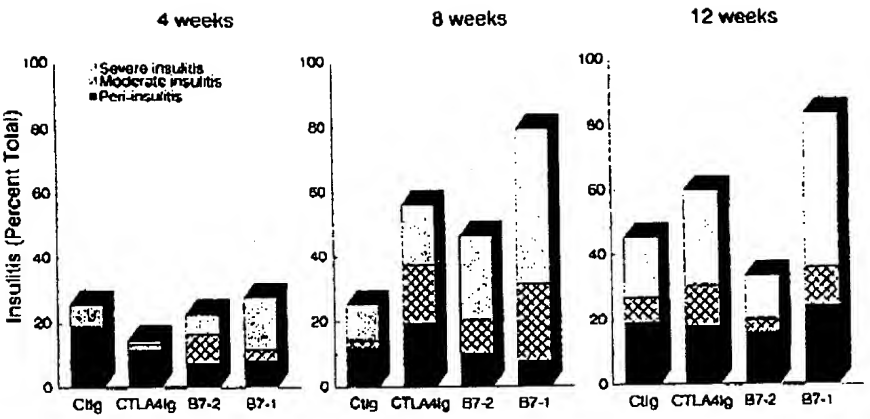

Figure 3. The incidence of insulitis in NOD mice treated with CD28 antagonists. Female animals from the various treatment groups were killed at 4, 8, and 12 wk of age. Pancreatic sections were stained with H&E and then scored for the presence of periinsulitis (*solid bars*), moderate insulitis (*cross-hatched bars*), or severe insulitis (*shaded bars*). Three to six animals (at least 50 islets) from each time point were scored. Insulitis is reported as the percentage of infiltrated islets per total islets scored for each treatment group and time point.

bination of anti-B7-1 and anti-B7-2 mAbs was more immunosuppressive than either drug alone (Zeng, J., et al., manuscript submitted for publication). Therefore, we examined the ability of the combined therapy to block the development of diabetes in NOD mice. 2–3-wk-old mice were treated with both anti-B7-2 and anti-B7-1 (16-10A1) mAbs as described in Materials and Methods. Despite the ability of anti-B7-2 mAb to inhibit disease onset, treatment with a combination of anti-B7-1 and anti-B7-2 mAbs resulted in an accelerated onset on disease (Fig. 4). By 12 wk of age, >65% of the combined treated female mice developed diabetes, and by 16 wk of age, all but one animal were hyperglycemic. Treatment of 2-wk-old NOD mice with the anti-B7-1 mAb (16-10A1) alone also made the disease worse (Fig. 4). In fact, hyperglycemia was detected in some mice as early as 8 wk of age in both treatment groups. More than 80% of the anti-B7-1–treated female mice were diabetic by week 12, and by 16 wk of age, 100% of the female mice were hyperglycemic. In contrast, at 16 wk of age only 20% of the CtIg-treated animals were diabetic, with only 70% developing diabetes by 24 wk of age. To eliminate the possibility that this phenomenon was due to nonspecific toxicity of the 16-10A1 mAb, these studies were repeated with another anti-B7-1 mAb, 1G10. As shown in Fig. 5 *A*, treatment of the female NOD mice with 1G10 also accelerated the development of diabetes. 100% of the 1G10-treated female mice became diabetic by week 18. Even more striking than the exacerbation of disease in the female mice was the observation that anti-B7-1 treatment induced disease in normally resistent male NOD mice (Fig. 5 *B*). Disease was first detected in these anti-B7-1– (16-

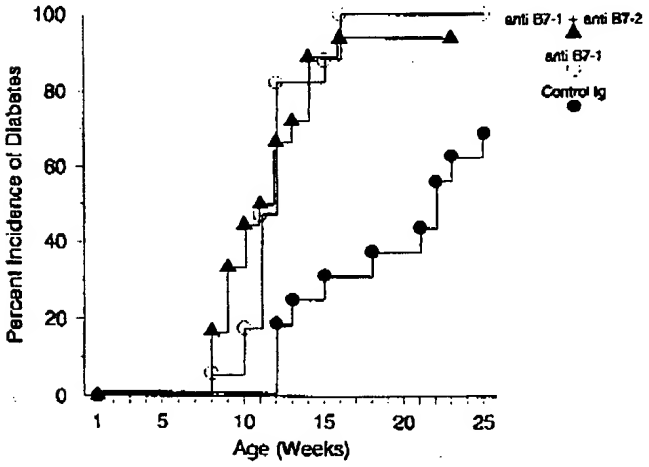

Figure 4. A combination of anti-B7-1 (16-10A1) and anti-B7-2 mAbs accelerates the onset of diabetes. Female NOD mice were treated at 2 wk of age with control Ig (; n = 16), anti-B7-1 (○; n = 17), or both anti-B7-1 and anti-B7-2 mAbs (▲; n = 18) as described. The treated animals were then monitored for the development of diabetes.

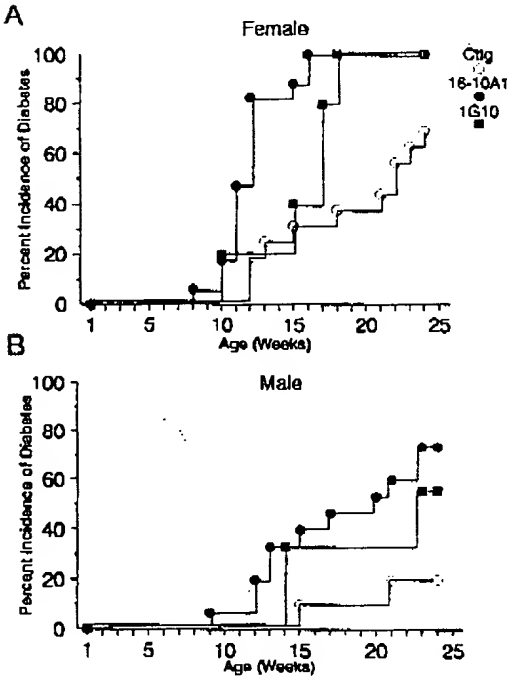

Figure 5. Anti-B7-1 therapy accelerates disease in both male and female NOD mice. Female (*A*) and male (*B*) NOD mice were treated with control Ig (○; female n = 16, male n = 10) or one of two anti-B7-1 mAbs: 16-10A1 (●; female n = 17, male n = 15) or 1G10 (■; female n = 5, male n = 9) beginning at 2 wk of age as described and followed for the development of diabetes.

10A1)–treated male mice at 8 wk of age. By 24 wk of age, 73% of these male mice had developed diabetes, while there was autoimmune diabetes in only 20% of the control-treated mice. Furthermore, 1G10 treatment resulted in the development of diabetes in >50% of the male NOD mice (Fig. 5 *B*). Finally, as seen with CTLA4Ig, treatment of NOD mice after 10 wk of age with anti-B7-1 mAbs did not alter the development of diabetes (data not shown).

Treatment of NOD Mice with Anti-B7-1 mAbs Alters Early Events in Disease Development. The development of insulitis was also examined in these anti-B7-1–treated animals. While all of the treatment groups exhibited insulitis at 8 wk of age in the anti-B7-1–treated mice, the islets appeared to be more severely infiltrated, with few if any intact islets remaining (data not shown). Further analysis of these mice revealed that anti-B7-1 treatment increased the time course and severity of insulitis in both male and female mice. As seen in Fig. 3, while anti-B7-1–treated female mice did not exhibit an overall increase in the percentage of islets affected at 4 wk of age, the severity of the infiltrate was increased (CtIg [mean clinical score = 7.45] and anti-B7-1 mAb [mean clinical score = 10.35]). By 8 wk of age, the effect was even more dramatic, with >70% of the islets from anti-B7-1–treated mice demonstrating moderate to severe insulitis. In fact, >50% of the islets had a severe insulitis, resulting in a complete loss of the islet architecture, compared with 10–26% in the other treatment groups (Fig. 3). While at 12 wk of age the percentage of islets infiltrated in the other groups was nearing that of the anti-B7-1–treated group, the severity of the infiltrate in the anti-B7-1 mAb–treated mice (mean clinical score = 41.33) was much greater than either the CtIg (mean clinical score = 16.28) or the CTLA4Ig (mean clinical score = 24.0) treated mice. A similar increase in the severity of insulitis was also observed in the anti-B7-1–treated male mice (data not shown).

We also examined the effects of anti-B7-1 mAb treatment in normal B6 mice. 2-wk-old animals were treated as previously described with anti-B7-1 mAb (16-10A1). Anti-B7-1–treated male and female B6 mice were monitored for 30 wk for the development of diabetes or insulitis. None of the treated animals developed any signs of diabetes, including insulitis. 12-wk-old B6 mice treated with either CtIg (*a*) or anti-B7-1 (16-10A1) mAb (*b*) demonstrated no signs of lymphocytic infiltrates into either the islets or pancreas (Fig. 6). In contrast, the islets of a 12-wk-old NOD mouse treated with anti-B7-1 (*d*) displayed severe infiltrate into all of the islets present within the pancreas. CtIg-treated NOD mice (*c*) also exhibited signs of lymphocytic infiltrate, although once again, not as severe as in the anti-B7-1–treated animals. Therefore, the anti-B7-1 mAb did not induce disease in the absence of the genetic predisposition.

Finally, we examined the cellular makeup of the pancreatic infiltrate in the various treatment groups. Pancreases from treated animals were isolated at 11–13 wk of age, and the infiltrating lymphocytes were examined. Both T and B cells were present in all of the groups, with 12–20% of the infiltrate being composed of $B220^+$ cells and 30–42% of it composed of $Thy1^+$ cells (data not shown). The CD4/CD8 ratios were also similar between all of the groups. Analysis of T cell activation by CD69 expression (50–52) demonstrated that T cells isolated from the pancreases of all of the female treatment groups were activated to some degree (mean fluorescent intensity [MFI] of control-treated animals = 10.85) compared with either splenic T cells from the same animals (MFI of control animals = 3.39) or age-matched male mice (MFI of control animals = 4.79). However, CD69 expression of pancreatic T cells isolated from both the male and female anti-B7-1–treated mice was significantly increased above the levels observed in the CtIg-, CTLA4Ig-, or anti-B7-2–treated animals (Fig. 7). The female anti-B7-1–treated mice exhibited a mean fluorescence intensity of nearly 2.5 times that of CtIg-treated female mice, and the male mice expressed levels 1.75 times that of CtIg-treated male mice. While Fig. 7 suggests that treatment with anti-B7-2 mAb resulted in a reduced expression of CD69 in the T cells isolated from the spleen or male pancreases, this was not observed in repeated experiments. The increased expression of CD69 on the B7-1–treated pancreatic T cells together with the increased severity of insulitis indicate that anti-B7-1 treatment alters a very early event in the disease process, resulting in an accelerated onset of disease.

Discussion

One model for the induction of T cell tolerance suggests that the inability of nonconventional APC to fully activate T cells due to their lack of costimulatory molecules results in T cell inactivation (anergy) and, in some instances, cell death. While several studies have demonstrated that the ectopic expression of MHC molecules and the appropriate costimulatory molecules on nonconventional cells, such as the islets of Langerhans, can induce an autoimmune destruction of the islets (17–19), few studies have directly examined the role of costimulatory signals during the normal development of autoimmune diabetes. In this study, we examined the role of the CD28/B7 signaling pathway in the generation and propagation of autoimmune diabetes in the NOD mouse model.

CTLA4Ig, a soluble CD28/B7 antagonist, has been shown to inhibit a variety of responses, including allogeneic and xenogeneic transplant rejection (45, 46), antibody responses (44), and autoimmune disease (53). In this study, CTLA4Ig treatment of NOD mice also resulted in a profound inhibition of disease onset when administered just before or at the onset of insulitis. Only 11% of the CTLA4Ig-treated mice became diabetic. Similar results were observed with an anti-B7-2 mAb, GL1, previously shown to be the dominant costimulatory CD28 ligand in allogeneic responses (33). Anti-B7-2 mAb treatment of NOD mice inhibited diabetes when initiated at 2 wk of age, with only 45% of the anti-B7-2–treated mice becoming diabetic compared with 87% of control treated mice. In both instances, the CD28 antagonists had no effect on the development of disease if administered late (>10 wk of age). These results suggest that these inhibitory reagents block early events in disease development. The

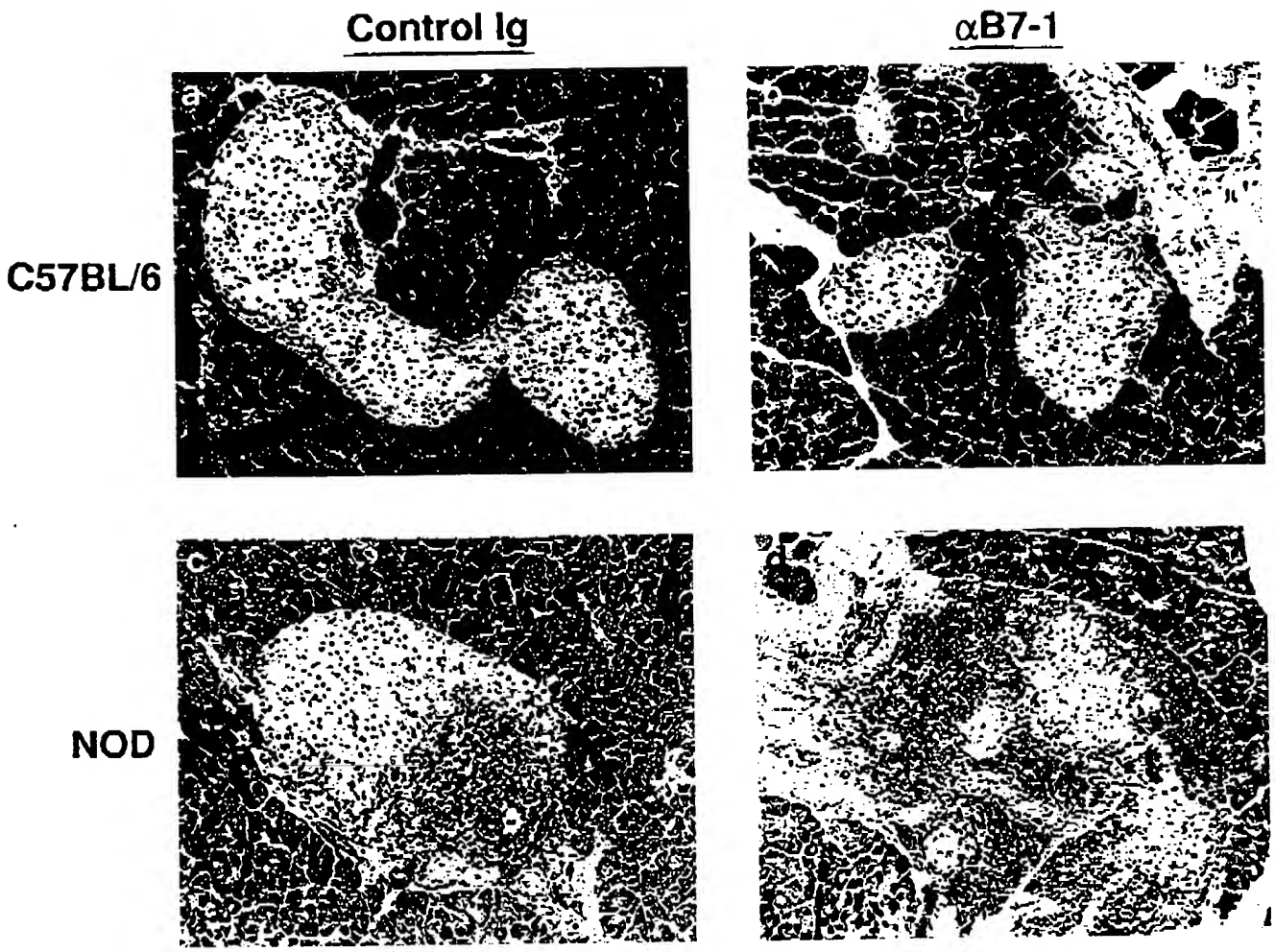

Figure 6. Anti-B7-1 treatment only induces insulitis in genetically susceptible strains of mice. NOD and B6 mice were treated with either anti-B7-1 (16-10A1) or a CtIg beginning at 2 wk of age as described in Materials and Methods. Representative H&E sections of 12-wk-old mice are shown. (*a*) CtIg-treated B6, (*b*) anti-B7-1–treated B6, (*c*) CtIg-treated NOD, and (*d*) anti-B7-1–treated NOD. ×200.

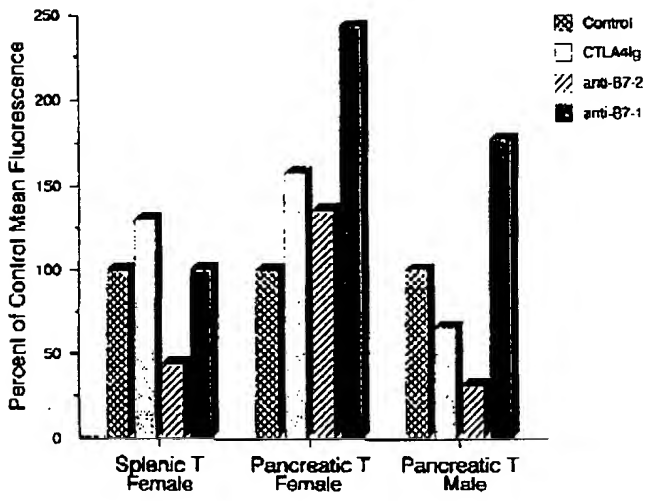

Figure 7. Phenotypic analysis of lymphocytes infiltrating the pancreas of treated animals. T cells isolated from either the spleen or pancreas of 11–13-wk-old treated mice were analyzed for surface expression of CD69 by FACS® analysis. The percentage of control mean fluorescence = (MFI of treatment group/MFI of control group) × 100. The MFI for the control-treated animals are female splenic T cells = 3.39, female pancreatic T cells = 10.85, and male pancreatic T cells = 4.79. Each group is composed of five mice, and the data are representative of two experiments.

inability of CTLA4Ig to block the development of diabetes when administered late in the disease process differs from the observations of Finck et al. (53) that late treatment could suppress an active autoimmune response in a model for murine lupus. This difference may be due to the more predominant role of antibodies in the lupus model or a difference in reagents, since in these studies only murine and not human CTLA4Ig had a beneficial effect.

While both CTLA4Ig and anti-B7-2 mAb treatment were able to decrease the incidence of diabetes, they had little effect

on the occurrence of insulitis. CTLA4Ig- and anti-B7-2–treated animals developed an equivalent lymphocytic infiltrate to that of CtIg-treated animals, so that by 12 wk of age, the mean clinical scores were essentially equivalent. Similar results were also obtained in treated male mice, although a significant degree of infiltration (>40% of the islets) was not detected until 12 wk of age (data not shown). Isolation of the lymphocytes infiltrating the pancreas demonstrated that equivalent numbers of T cells (CD4 and CD8) and B cells were present in all three groups. Moreover, the degree of T cell activation, as assessed by the expression of CD69, was equivalent in the CtIg-, CTLA4Ig-, and anti-B7-2–treated groups. Therefore, quantitatively, the infiltrate appears to be quite similar between CtIg-treated animals that develop disease and CTLA4Ig- or anti-B7-2–treated animals that do not. However, qualitative differences in the infiltrate may exist. One possibility is that the interruption of critical interactions between CD28 and its costimulatory ligands may result in the induction of anergy to islet antigens such as GAD65. These antigens have been shown to play an important role in the early phase of the development of disease (11, 12). Alternatively, CTLA4Ig and anti-B7-2 treatment may inhibit disease, not by inducing anergy to these islet antigens, but by altering the balance of Th1 and Th2 cells that infiltrate the islets and respond to the autoantigens (54). In fact, recent data from Kuchroo et al. (54a) have suggested that the in vivo functional effects of anti-B7-2 mAbs in an experimental autoimmune encephalomyelitis (EAE) model are a result of changes in the balance of Th1 and Th2 subsets in these animals.

While anti-B7-2 treatment inhibited diabetes development in NOD mice, its effects were not as profound as CTLA4Ig treatment. This could be due to the differences in affinity of the mAb and CTLA4Ig for B7-2 or CTLA4Ig's ability to bind to alternative ligands, such as B7-1. Therefore, NOD mice were treated with either a combination of anti-B7-1 plus anti-B7-2 mAbs or anti-B7-1 alone. In contrast to the immunosuppression of NOD disease observed after anti-B7-2 or CTLA4Ig therapy, treatment of NOD mice with anti-B7-1 mAbs at the onset of insulitis resulted in a more severe infiltrate and a rapid onset of disease in both male and female mice. This effect was observed with two different anti-B7-1 mAbs, 16-10A1 and 1G10, even though 1G10 has a 40-fold lower avidity for B7-1 than does 16-10A1 (33). Unlike the CTLA4Ig or anti-B7-2 treatment, which had little effect on the development of insulitis, the treatment of NOD mice with anti-B7-1 mAbs resulted in a more severe and rapid onset of insulitis. Furthermore, the T cells isolated from the anti-B7-1–treated female and male mice expressed higher levels of CD69, indicating they were more highly activated than T cells isolated from the other treatment groups.

These results indicate that B7-1 plays a direct role in controlling this autoimmune response by directly signaling through the B7-1 molecule, interrupting a critical interaction between B7-1 and one of its ligands, or interacting with a distinct population of APC during the development of disease. Interestingly, transfectants of both B7-1 and B7-2 are capable of providing the necessary costimulatory signals to the T cell (37–39). However, differences in both the expression and function of these two molecules have been observed. The expression of B7-2 occurs much more rapidly than B7-1 after B cell activation (33, 41). Furthermore, Ig cross-linking only induces significant levels of B7-2 and not B7-1 (40). Thus, it is possible that B7-2 is expressed on APC essential for initiating full-blown diabetes, while B7-1 is expressed on cells that regulate the development of insulitis. For instance, B7-1 is expressed on activated T cells (55). Thus, the anti-B7-1 mAb might deliver a signal to the T cells that alters effector cell function, such as lymphokine production, resulting in a potential shift in the balance of Th1 and Th2 subsets. Alternatively, the interaction of the anti-B7-1 mAb with conventional APC could increase the antigen presentation or costimulation capabilities of the cells, resulting in a more potent T cell response. Finally, anti-B7-1 treatment may mediate its effects by blocking the interaction of B7-1 with one of its counter-receptors, CTLA-4 (43). Recent data from our laboratory suggest that the signals delivered to the T cell by CD28 and CTLA-4 may be different. F(ab) fragments of anti-CTLA-4 antibodies augment T cell proliferation in an allogeneic MLR by blocking an off signal presumably delivered by a CTLA-4 ligand (42). These results suggest the possibility that while the CD28 molecule provides important costimulatory signals to the T cell, CTLA-4/B7-1 interactions may actually function to downregulate an immune response. The interruption of such a negative signal by anti-B7-1 mAbs would prevent the downregulation of an autoimmune response and result in a more severe disease.

Despite the ability of anti-B7-2 mAb to inhibit costimulation and prevent diabetes, a combination of anti-B7-1 and anti-B7-2 mAbs increased the onset of diabetes in both female and male mice (data not shown), similar to anti-B7-1 mAb treatment alone. These results raise the possibility that B7-1 and B7-2 function at different time points during the development and propagation of this autoimmune response. In this regard, there is good evidence that this disease progresses in at least two stages (56). The first event results in the development of insulitis, and later events are responsible for the progression to full-blown diabetes. While both CTLA4Ig and anti-B7-2 treatment inhibited the development of diabetes, neither treatment prevented the occurrence of insulitis. Furthermore, animals not receiving the additional three doses at weeks 6, 7, and 8 were not protected from developing diabetes (data not shown). Together, these results suggest that anti-B7-2 and CTLA4Ig treatment act late in disease development. In contrast, anti-B7-1 mAbs increased both the rate and severity of insulitis, and the additional three doses at weeks 6, 7, and 8 were not necessary for exacerbation of disease (data not shown), suggesting that this therapy altered the initial stages of the disease process. There are several possible explanations for the exacerbation of disease observed with a combination of anti-B7-1 and anti-B7-2 mAbs. First, it is possible that the initial activation event may be CD28 independent. If this is the case, then this event would rely on alternative costimulatory pathways and would therefore not be affected by blockade of the CD28 ligands, B7-1 or B7-2.

In this regard, it is interesting to note that early alloantigen responses are largely unaltered in vivo or in vitro in CD28-deficient mice (57). By comparison, the later events of disease progression would appear to be exclusively CD28 dependent. Alternatively, all of the stages of autoimmune diabetes may be CD28 dependent, and the initiation of treatment at 2 wk of age is not early enough to prevent the development of insulitis, but would inhibit the later events responsible for disease progression. In either case, autoreactive T cells would be activated and express both CTLA-4 and B7-1. Therefore, the exacerbation of disease mediated by the anti-B7-1 mAb would dominate the inhibitory effects of anti-B7-2 treatment by either directly signaling through the B7-1 molecule or interrupting a critical interaction responsible for shutting down the immune response. Future experiments with Fab and F(ab)'2 fragments of the anti-B7-1 and anti-B7-2 mAbs, as well as genetically altered B7-1 and B7-2 knockout mice, will allow us to determine the mechanism by which B7-1 treatment exacerbates disease.

Finally, these observations do not appear to be restricted to the NOD autoimmune mouse model. Preliminary studies performed in collaboration with Dr. Steve Miller (Northwestern University, Chicago, IL) in an EAE model have shown that treatment of mice with anti-B7-1 during the primary response to proteolipid protein resulted in more rapid and severe secondary relapses (Miller, S., C. Vanderlugt, D. J. Lenschow, and J. A. Bluestone, unpublished observations). Therefore, the mechanism responsible for the anti-B7-1–mediated acceleration of disease in the NOD mouse model may be similar for other autoimmune diseases.

In conclusion, these results clearly demonstrate that T cell costimulation is an essential component of the in vivo activation of autoreactive T cells and the development of autoimmune diabetes. Thus, the manipulation of this costimulation pathway may provide a powerful new target for the development of future therapies for diabetes and other autoimmune diseases.

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Appendix II

Distinct Roles for B7-1 (CD-80) and B7-2 (CD-86) in th Initiation of Experimental Allergic Encephalomyelitis

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Abstract

The activation and differentiation of T cells require both antigen/MHC recognition and costimulatory signals. The present studies examined the role of B7-1 (CD80) and B7-2 (CD86) costimulation in the prototypic autoimmune disorder, experimental allergic encephalomyelitis (EAE). In adoptively transferred EAE, in vitro activation of myelin basic protein (MBP)-specific lymph node cells was inhibited by the combination of anti-CD80 plus anti-CD86, but not individually. However, in actively induced disease, one injecti n of anti-CD80 significantly reduced disease, while anti-CD86 exacerbated disease. Interestingly, one injection of CTLA-4Ig suppressed disease, while multiple injections resulted in enhanced disease. Thus, the costimulation provided by B7-1 molecules appears to be important for the development of encephalitogenic T cells. The enhanced disease caused by multiple injections of CTLA-4Ig or a single injection of anti-CD86 suggests an inhibitory function for CD86 interaction with its counterreceptors CD28 and CTLA-4 in EAE. Alternatively, these results are consistent with an essential timing requirement for the coordinated interaction of B7 and CD28 family receptors, and that disruption of this critical timing can have opposing results on the outcome of an immune response. (*J. Clin. Invest.* 1995. 96:2195–2203.) Key words: allergic encephalomyelitis • autoimmune disease • B7 • T lymphocyte • T cell costimulation

Introduction

For T cell activation to take place, it is necessary for the T cell to receive two signals from the antigen presenting cell (APC)[1] (1–3). One signal determines the antigen-specificity of the response and results from antigenic peptide bound to MHC interacting with the T cell receptor. The second signal, termed costimulation, is provided by accessory molecules on the APC and appears to be necessary for functional T cell activation (4). The B7 family of cell surface molecules expressed on APC is capable of providing this second signal to T cells via two receptors, CD28 and CTLA-4 (5, 6).

Previously, we have examined the role of the B7:CD28/CTLA-4 interaction in the induction of a prototypic, T cell mediated, autoimmune disorder experimental allergic encephalomyelitis (EAE) (7). EAE is induced by CD4+, class II MHC-restricted T cells of the Th1 phenotype that predominantly secrete the cytokines IL-2 and IFN-γ (8–10). In most murine models of EAE, the response of these encephalitogenic T cells is directed against either of two myelin antigens, MBP or proteolipid protein (11–12). We demonstrated that by using a fusion protein ligand for B7, CTLA-4Ig, we were able to inhibit the proliferation and IL-2 production of MBP-specific LNC during their activation in vitro, resulting in reduced clinical disease upon subsequent transfer (7).

It appears that Th1-like T cells are the inducing cells in the EAE model (8–10). As we and others have shown, costimulation provided through CD28 is very important for the secretion of cytokines by Th1 cells (4, 7, 13). On the other hand, Th2 cells, which predominantly secrete IL-4 and IL-10, are helper cells important for IgG1 and IgE antibody production and appear to be important regulatory cells in inflammatory, DTH-like responses, including EAE (10, 14, 15). The ability of CD28 to provide costimulation to Th2 cells is less well-defined, although there appear to be examples where B7 may also play a role in IL-4 production (16, 17).

At least two members of the B7 family of CD28 ligands have been defined, B7-1 (CD80) and B7-2 (CD86) (18–24). These molecules, although only having modest homology, are each able to provide costimulation to T cells for proliferation and IL-2 production (22, 25, 26). It is probably for this reason, that a mouse genetically deficient for CD80 (B7-1), was essentially immunocompetent (21). In addition, it is likely that there may be another member of the B7 family capable of providing a costimulatory signal to T cells through CD28 and/or CTLA-4 (27, 28).

B7-1 (CD80) and B7-2 (CD86) may be expressed differentially on various APC and their kinetics of expression and binding may also differ (24). B7-2 is constitutively expressed by monocytes, however B7-1 can be induced on these APC by IFN-γ (20, 29, 30). On B cell populations following an activation stimulus, B7-2 is expressed within 6 hr while B7-1 expression occurs significantly after that time (27, 31, 32). Interestingly, only B7-2 expression is increased on dendritic cells after exposure to IFN-γ (33).

Initial studies indicated that CTLA-4 provided a costimulatory signal in conjunction with CD28 (34). Recent evidence suggests that the signaling through the CTLA-4 molecule may

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1. *Abbreviations used in this paper:* APC, antigen presenting cell; EAE, experimental allergic encephalomyelitis; LNC, lymph node cells; MBP, myelin basic protein.

actually mediate a negative regulatory function. There are several lines of evidence to support this notion. First, in CD28-deficient mice, costimulation provided by B7$^+$ accessory cells did not appear to transduce a positive signal (35). Other studies provided direct evidence for the ability of CTLA-4 to deliver a negative signal (36, 37). Furthermore, constitutive expression of murine B7-1 (CD80) on mature B cells resulted in depressed antibody responses to T cell-dependent hapten-protein conjugates, suggesting that B7-1 may contribute to feedback inhibition of T cell-dependent immune responses in vivo (38).

In light of these differences, we examined the separate roles of the different costimulatory molecules B7-1 (CD80) and B7-2 (CD86) in a T cell-mediated, organ-specific model of autoimmunity, EAE. Our results indicate that antigen (MBP)-primed T lymphocytes are capable of becoming activated and encephalitogenic by in vitro stimulation utilizing either B7 ligand, CD80 or CD86. However, there appears to be differential effects of these ligands on the development of encephalitogenic T cells in vivo, demonstrated by different clinical outcomes when anti-B7 reagents are administered in actively induced EAE.

Methods

Mice. Female SJL/J and (PL × SJL)F1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6–8 wk of age. Mice were 10–12 wk of age when experiments were initiated. All procedures were in compliance with guidelines set by the NIH Animal Care and Use Committee.

Reagents. Whole MBP was prepared from guinea pig spinal cords (Rockland, Inc., Gilbertsville, PA) as previously described (39). Human CTLA-4Ig and a control fusion protein hIgG1 were prepared by utilizing a previously described strategy (40). αCD80 mAb 16-10A1 (41) was provided by Dr. Hans Reiser (Dana Farber Cancer Institute, Boston, MA). Anti-murine CD86 mAb GL-1 (23) was provided by Dr. Richard Hodes (National Institute of Aging, NIH, Bethesda, MD). mAb 9.3 was used as a control for αCD80 and a rat IgG2a antibody was used as a control for αCD86. All antibodies were purified from hybridoma culture supernatants and the endotoxin content of the hCTLA-4Ig, the control fusion protein, and all mAb used in these studies was less than 0.4 EU/mg. Fab fragments were made from anti-CD80, anti-CD86, and control antibodies using the ImmunoPure Fab Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The Fab fragments were subsequently run on a 12% SDS-PAGE gel under nonreducing conditions with molecular weight markers and whole antibody molecules. Gels were stained with Coomasie blue and purity assessed.

Induction of EAE. For adoptively transferred EAE, SJL mice were immunized with MBP (400 μg) in CFA and 10 d later draining lymph nodes removed. MBP-specific LNC were cultured in RPMI 1640 (Bio Whittaker, Walkersville, MD) supplemented with 10% FBS, penicillin G (100 U/ml), glutamine (2 mM), nonessential aminoacids, Hepes buffer, 2-ME, and MBP (25 μg/ml) for 4 d. LNC were then washed and adjusted to the required concentration in PBS so that each recipient received 3 × 10^7 cells in a 0.2 ml suspension via the tail vein. Recipient mice were examined daily for signs of disease by a blinded examiner and graded on the following scale: 0, no abnormality; 1, a limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia, or premoribund state (10).

For actively induced EAE, (PL × SJL)F1 mice were immunized subcutaneously with MBP/CFA (400 μg MBP/mouse) twice separated by one week. Animals were observed daily for signs of disease as above.

Lymphocyte proliferation. Proliferative responses were measured on MBP-specific LNC by incubating LNC (2 × 10^5 cells/well) with MBP (25 μg/ml) or medium alone in the presence or absence of the various concentrations of anti-B7 reagents as indicated. For MBP-specific T cell lines, T cells (1 × 10^5 cells/well) were cultured with irradiated (3000 rad), syngeneic splenocytes as APC (2 × 10^5 cells/well). T cell lines were generated as previously described (42). Cultures were maintained in 96-well, flat-bottom microtiter plates (Costar, Cambridge, MA) for 96 h at 37°C in humidified 5% CO_2 air. The wells were pulsed with 1 μCi/well of [^{3}H]methyl-thymidine (New England Nuclear, Boston, MA) for the final 16 h of culture. Cells were harvested on glass fibers and incorporated [^{3}H]methyl-thymidine was measured with a Betaplate counter (Wallac, Gaithersburg, MD). Results were determined as arithmetic means from quadruplicate cultures and SEM shown.

Measurement of lymphokine production. An IL-2–dependent cell line, CTLL.EV (43), was generously provided by Dr. W. Paul (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). 50 μl of supernatants from experimental cell cultures were assayed in quadruplicate. Results were compared with proliferation of the cell line to known amounts of IL-2 as standards. Standard deviation of replicate cultures was < 10% of the mean.

Statistical methods. Treatment effects were assessed by the Mann-Whitney Sum of Ranks test.

Results

Effects of anti-B7 reagents on MBP-specific T cell proliferation and IL-2 production. Previously, we demonstrated that the presence of CTLA-4Ig during the in vitro activation stage of adoptively transferred EAE resulted in a decreased proliferative response by MBP-specific LNC, reduced IL-2 production, and a diminution of clinical disease on subsequent transfer (7). With antibodies that specifically recognize two members of the B7 family, CD80 (B7-1) and CD86 (B7-2), we were able to examine the ability of these molecules to provide costimulation to MBP-primed LNC. As shown in Fig. 1, neither the presence of αCD80 nor αCD86 alone substantially inhibited the proliferative response of MBP-specific LNC to MBP. However, the addition of both αCD80 plus αCD86 resulted in dramatic inhibition of T cell proliferation (81% inhibition at the concentration of 0.625 μg/ml of both antibodies). As we had shown previously, addition of CTLA-4Ig inhibited T cell proliferation in a dose-dependent manner (Fig. 1). Of note, while increasing the concentration of CTLA-4Ig resulted in decreased proliferation, the combination of αCD80 and αCD86 was most efficient at inhibiting T cell proliferation at the lowest concentration examined. This unexpected dose-response suggested the possibility that signal transduction through the B7 receptor is induced by the antibodies at lower concentrations, followed by effects consequent to receptor blockade at higher concentrations. To test this possibility, Fab fragments of the αB7 antibodies were used to examine their effect on T cell proliferation. Similar to CTLA-4Ig, use of Fab fragments resulted in a dose-dependent inhibition of T cell proliferation (Fig. 1 *C*).

In addition to T cell proliferation, we also examined the secretion of IL-2 into the tissue culture supernatants (Fig. 2). Since a low concentration of αCD80 plus αCD86 was most effective in inhibiting T cell proliferation, we used these reagents at an optimal concentration of 1 μg/ml when examining IL-2 production. For comparison, CTLA4-Ig was used at a concentration of 5 μg/ml. Both the combination of αCD80 plus αCD86 or CTLA4-Ig alone inhibited the production of IL-2 by MBP-specific LNC. It is important to note that in our previous study, the dose response for the effect of CTLA-4Ig on IL-2 production by MBP-specific LNC reached a plateau at 5 μg/ml, such that IL-2 production was inhibited to a similar degree by CTLA-4Ig when used at a concentration of 30 μg/ml (7).

Because of the decreased proliferative response and reduced

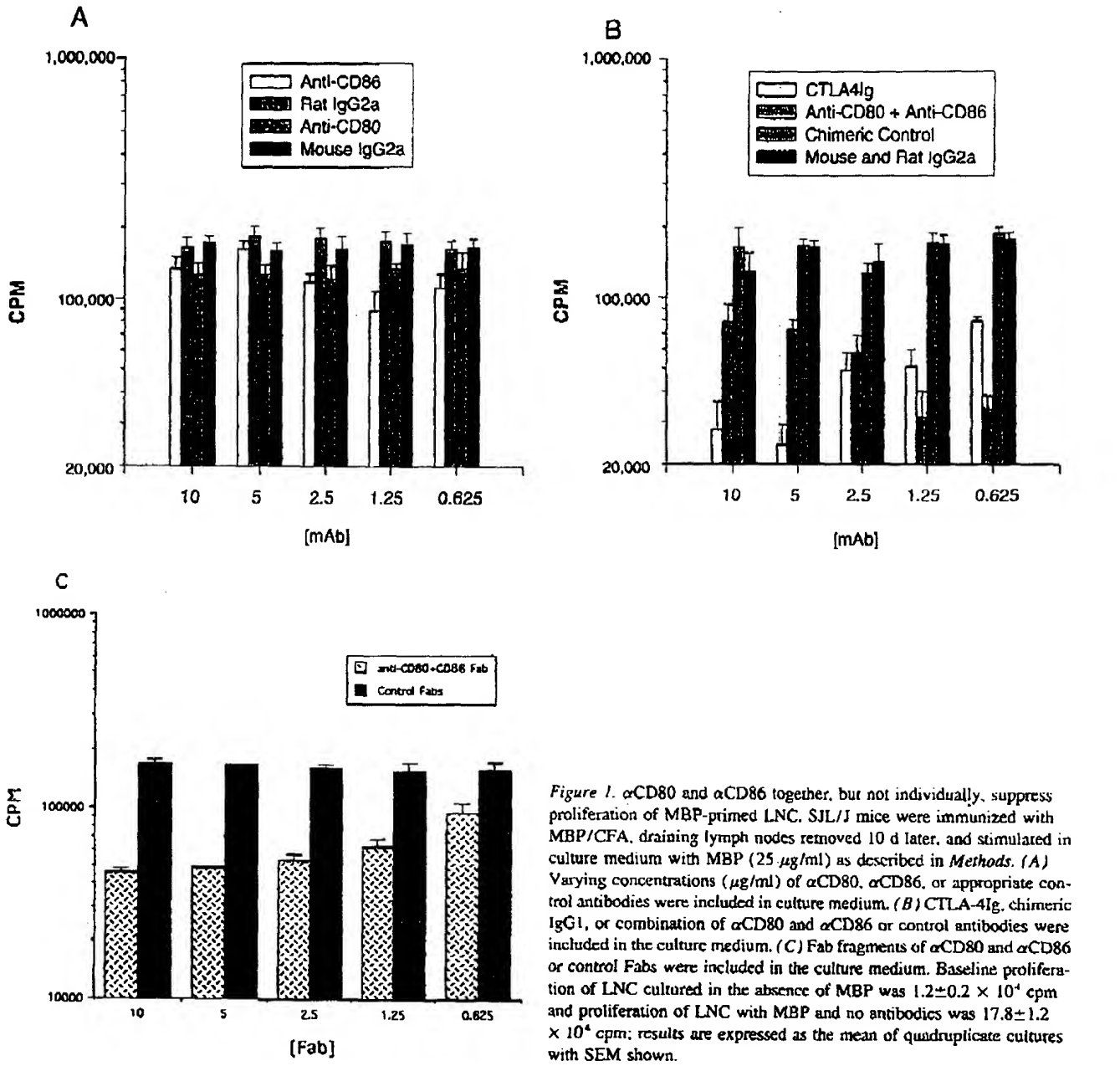

Figure 1. αCD80 and αCD86 together, but not individually, suppress proliferation of MBP-primed LNC. SJL/J mice were immunized with MBP/CFA, draining lymph nodes removed 10 d later, and stimulated in culture medium with MBP (25 μg/ml) as described in *Methods*. *(A)* Varying concentrations (μg/ml) of αCD80, αCD86, or appropriate control antibodies were included in culture medium. *(B)* CTLA-4Ig, chimeric IgG1, or combination of αCD80 and αCD86 or control antibodies were included in the culture medium. *(C)* Fab fragments of αCD80 and αCD86 or control Fabs were included in the culture medium. Baseline proliferation of LNC cultured in the absence of MBP was $1.2\pm0.2 \times 10^4$ cpm and proliferation of LNC with MBP and no antibodies was $17.8\pm1.2 \times 10^4$ cpm; results are expressed as the mean of quadruplicate cultures with SEM shown.

IL-2 production, the proliferative response of the above T cell lines upon restimulation with MBP was examined. Mice were immunized with MBP in the absence of costimulatory blockade, and the draining LNC cultured in the presence of CTLA-4Ig or anti-CD80 plus anti-CD86. Despite reduced IL-2 production in the presence of CTLA-4Ig or αCD80 plus αCD86 during the initial stimulation in vitro (Fig. 2), all cell lines responded to MBP restimulation and produced significant amounts of IL-2 (Fig. 3). Addition of CTLA-4Ig during the second stimulation of these lines resulted in no or only modest inhibition (< 25%) of the proliferation of cells previously activated in the presence of the chimeric IgG1 or CTLA-4Ig (Fig. 3 *A*). The addition of αCD80 plus αCD86 during the second stimulation did not significantly inhibit T cell proliferation (Fig. 3 *B*). This suggests that the lack of B7 costimulation did not tolerize the entire population of MBP-specific T cells. Alternatively, because these reagents only block B7 interactions with CD28 and CTLA-4, other costimulatory pathways were still intact and able to mediate the induction of MBP-specific T cells (44, 45). The B7 blocking reagents were present during the entire 10-d period of culture following the initial antigenic stimulation, in order to prevent costimulation in trans from APC present during this period. It should also be noted that our previous study showed that when CTLA-4Ig was present during both in vivo priming and in vitro culture that subsequent MBP-induced LNC proliferation and IL-2 secretion were essentially completely blocked (7).

The ability of either CTLA-4Ig or αCD80 plus αCD86 to inhibit the proliferative response of long-term MBP-specific T cell lines was also examined. Unlike the marked inhibition seen with MBP-specific LNC, the proliferative response of the T cell lines was only minimally inhibited (< 15% inhibition, data not

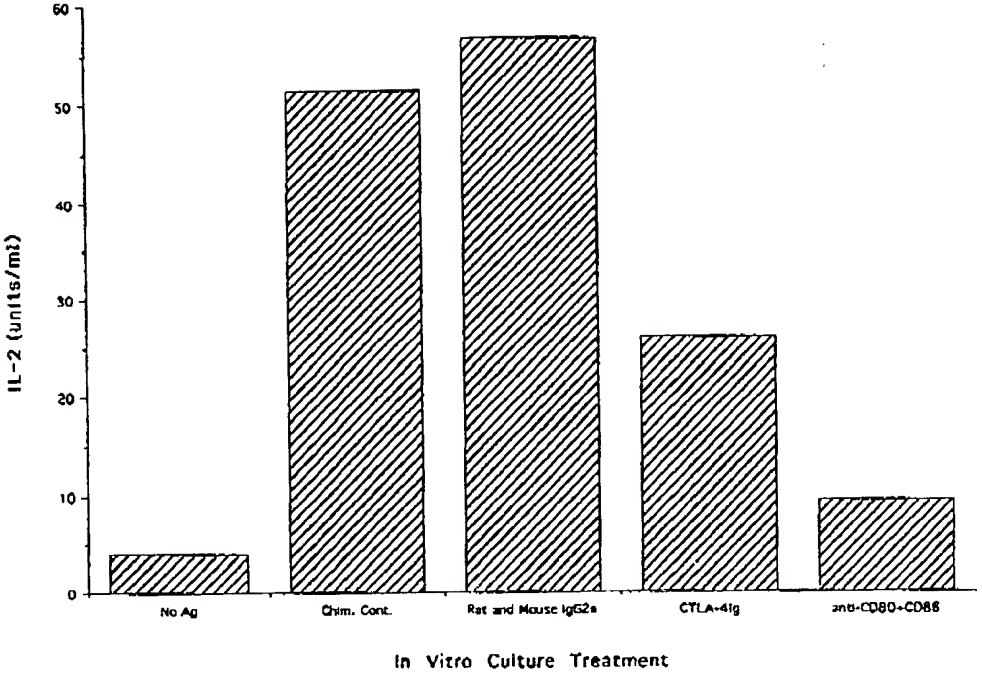

Figure 2. αCD80 and αCD86 together suppress IL-2 secretion in cultures of MBP-specific LNC. MBP-specific LNC were obtained as described in *Methods* and cultured with MBP (25 μg/ml) in the presence of CTLA-4Ig (5 μg/ml), chimeric IgG1 (5 μg/ml), αCD80 and αCD86 (1 μg/ml each), or the appropriate control antibodies. Cells were also cultured in the absence of additional MBP. Supernatants were harvested after 20 h. IL-2 levels were determined using the IL-2–dependent cell line, CTLL.EV. Results are representative of several independent experiments. Carry over of antigen from the in vivo priming accounts for the IL-2 secretion in the no Ag cultures, as LNC from unprimed mice did not secrete IL-2.

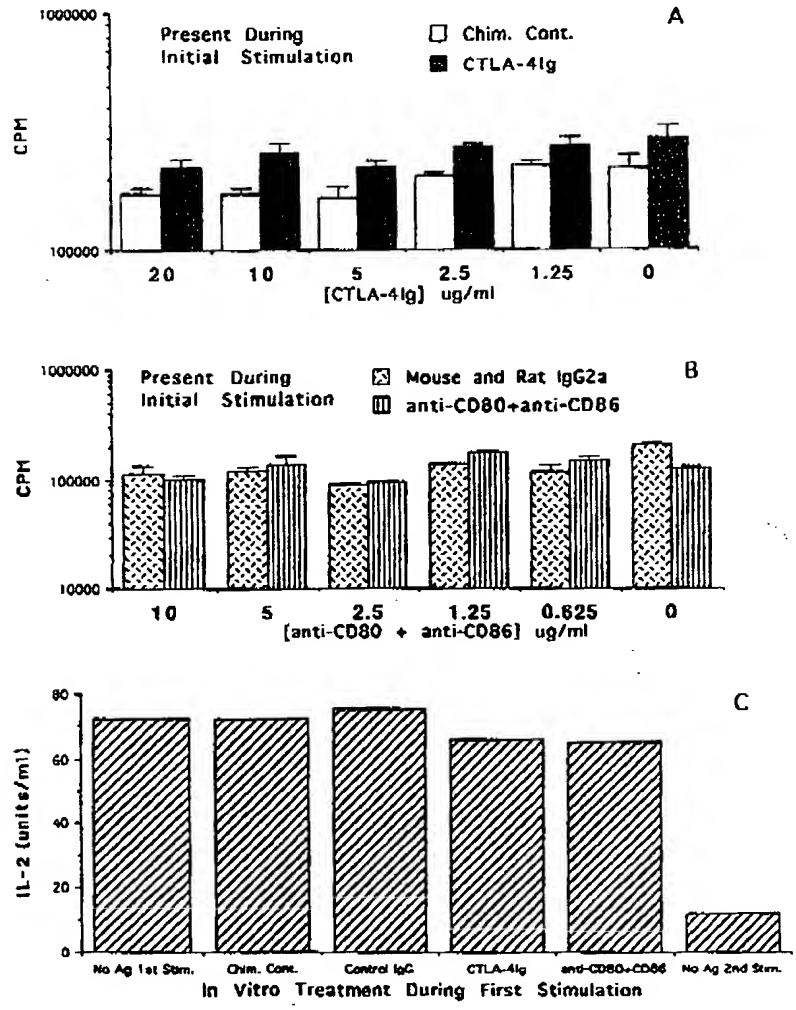

Figure 3. Antigenic restimulation of MBP-specific LNC initially stimulated in the presence of anti-B7 reagents. Mice were primed with MBP in the absence of costimulatory receptor blockade, and draining LN harvested on day 10. MBP-specific LNC were then stimulated in vitro in the presence of αCD80 and αCD86 or CTLA-4Ig as described in Fig. 2. Following the initial stimulation, T cell lines were cultured with syngeneic, irradiated splenocytes as previously described (42). Cells were cultured with the same concentration of either αCD80 and αCD86 (1 μg/ml) or CTLA-4Ig (5 μg/ml) for 10 d. Cell lines were then restimulated with MBP (25 μg/ml) and various concentrations of anti-B7 reagents. In *A*, MBP-specific LNC initially stimulated in the presence of chimeric IgG1 or CTLA-4Ig were restimulated in the presence of MBP and various concentrations of CTLA-4Ig as indicated. In *B*, MBP-specific LNC initially stimulated in the presence of αCD80 and αCD86 or control antibodies were restimulated in the presence of MBP and various concentrations of αCD80 and αCD86 as indicated. In *A* and *B*, baseline proliferation of cell lines in the absence of MBP was under 5×10^3 in all cases. In (C), MBP-specific LNC were initially stimulated with MBP in the presence of the following reagents: CTLA-4Ig (5 μg/ml), chimeric IgG1 (5 μg/ml), αCD80 and αCD86 (1 μg/ml), or control antibodies. Upon restimulation with MBP, after 20 h, supernatants were harvested and IL-2 levels determined using the CTLL.EV cell line. IL-2 production by cells initially stimulated with MBP in the presence of chimeric IgG1, but not receiving antigenic restimulation is also shown.

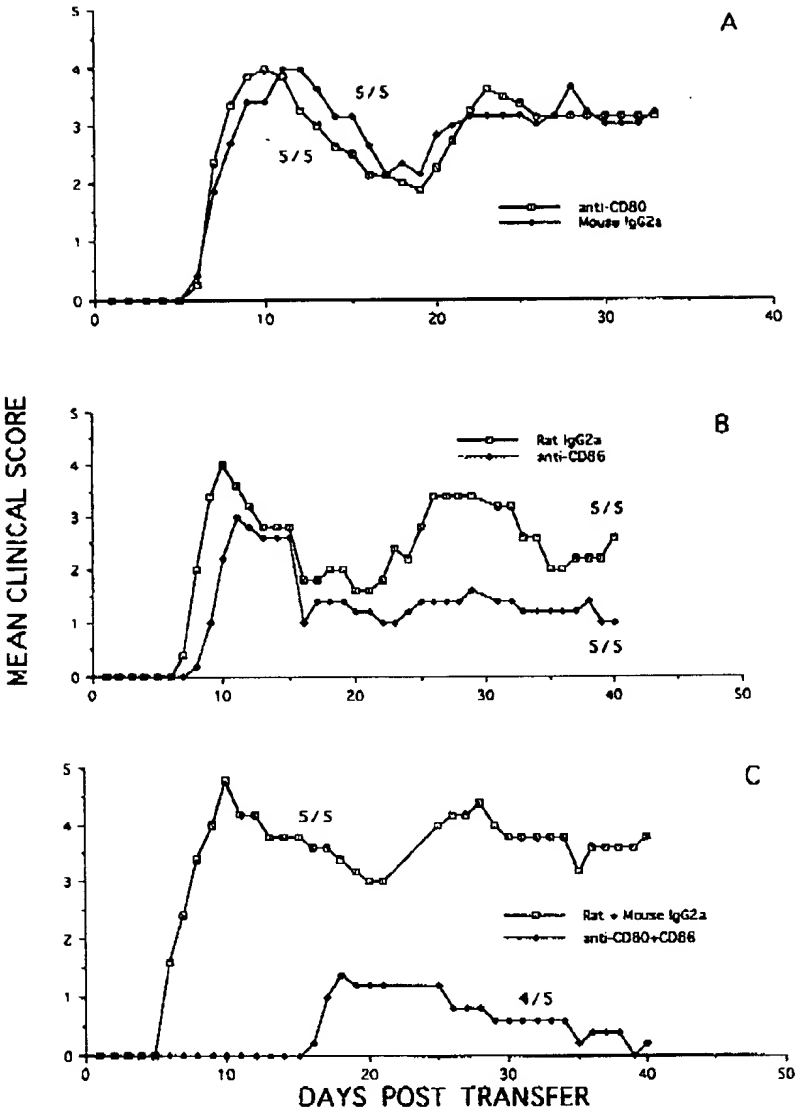

Figure 4. Distinct effects of addition of anti-B7 reagents during in vitro activation of MBP-specific LNC. LNC obtained 10 d after immunization with MBP/CFA were activated in vitro with MBP (25 μg/ml) and anti-B7 antibodies. LNC were activated in the presence of anti-CD80 or mouse IgG2a control mAb (5 μg/ml) *(A)*, anti-CD86 or rat IgG2a control antibody (5 μg/ml) *(B)*, or anti-CD80 plus anti-CD86 or control antibodies (1 μg/ml each) *(C)*. 30 million cells were injected i.v. into naive recipients on day 0. Mice were examined daily and a mean clinical score was assigned for each group of five mice. In *A*, there was no difference in disease course between the two groups of mice ($P > 0.05$). Significant differences between groups were noted in *B* for days 21–40 ($P < 0.01$) and in *C* for days 1–40 ($P < 0.01$). The incidence of clinical disease during the observation period is indicated next to the corresponding clinical course.

shown). This is consistent with the results above and prior reports suggesting that B7 costimulation is most important for naive T cells (46, 47). Furthermore, these results are consistent with those of others which suggest that the priming of Th2 cells is more dependent upon B7 costimulation than Th1 cells (16, 48).

Adoptively transferred EAE with T cells that have been activated in vitro in the presence of αCD80 and/or αCD86. Although either αCD80 or αCD86 alone did not inhibit MBP-specific T cell proliferation in vitro, the possibility existed that one of these ligands specifically provided an important costimulatory signal necessary to determine encephalitogenicity. As shown in Fig. 4, the presence of either αCD80 or αCD86 alone (10 μg/ml) during the activation of MBP-specific LNC in vitro did not result in a reduction in the severity of transferred EAE during the first episode of disease. Interestingly, while the relapse was quite similar in groups that received anti-CD80 and control antibody (Fig. 4 *A*), the relapse (days 25–35 posttransfer) was much less severe in mice that received MBP-specific LNC activated in the presence of anti-CD86 (Fig. 4 *B*). However, consistent with the observations in vitro, the presence of both αCD80 and αCD86 resulted in a delay in the onset and reduction in the severity of clinical disease (Fig. 4 *C*). The near complete abrogation of adoptively transferred EAE in mice receiving cells activated in the presence of anti-CD80 plus anti-CD86 is consistent with our previous results observed with CTLA-4Ig (7).

In vivo administration of CTLA-4Ig, αCD80 and/or αCD86 in actively induced EAE. Although both CD80 and CD86 appear to be able to provide costimulation to encephalitogenic T cells in vitro, because these molecules are differentially regulated on various APC populations, we examined the effect of CTLA-4Ig, αCD80 and/or αCD86 on actively induced EAE. For disease induction, a model was chosen where (PL × SJL)F1 mice were immunized twice with MBP/CFA separated by one week. This regimen was chosen to avoid the confounding effects of pertussis toxin administration on disease induction (49, 50).

In our initial experiment, CTLA-4Ig or chimeric control IgG1 was administered every other day for 10 injections (100 μg each injection i.p.), beginning the day before the first immunization. As shown in Fig. 5, not only was clinical disease not suppressed, there was a substantial increase in disease from

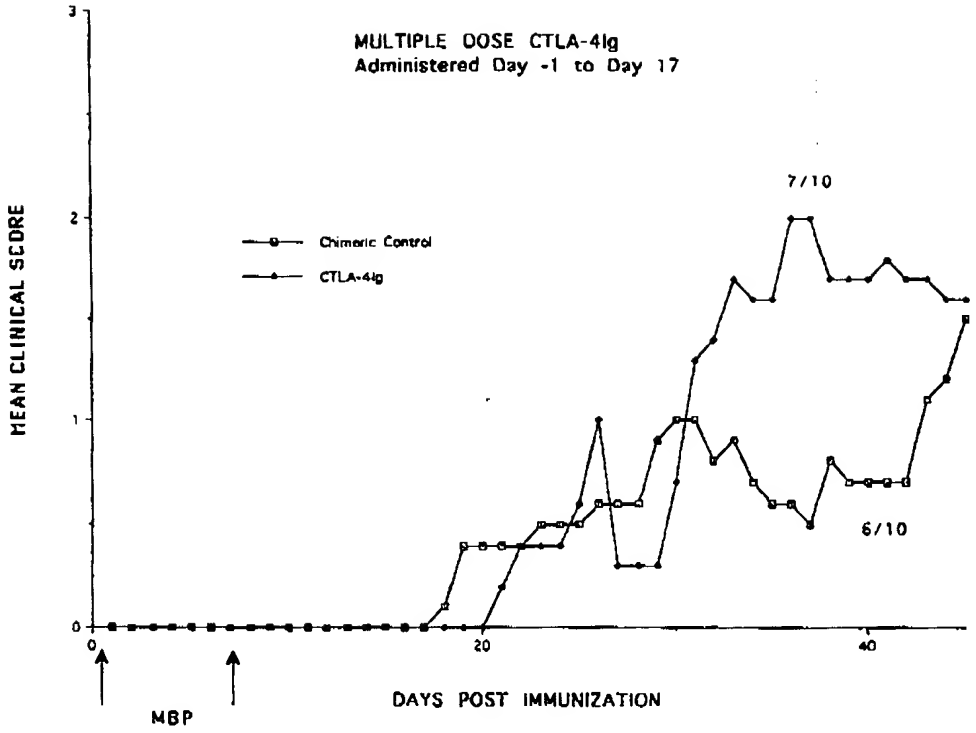

Figure 5. Multiple injections of CTLA-4Ig enhances actively induced EAE. Groups of 10 (PL × SJL)F1 mice were immunized on day 0 and day 7 with MBP/CFA (indicated by *arrows*). Beginning on day -1, mice received either CTLA-4Ig or chimeric IgG1 (100 μg) every other day for a total of 10 injections. Mice were examined daily and a mean clinical score assigned. Comparing the two groups of mice for the entire course of disease, there was no significant difference [days 1–45, ($P > 0.05$)], however disease was significantly augmented for days 25–45 ($P < 0.01$). The incidence of clinical disease during the observation period is indicated next to the corresponding clinical course.

days 30–45 postimmunization in the CTLA-4Ig-treated group ($P < 0.01$).

We next examined the effect of one injection of CTLA-4Ig, αCD80 and/or αCD86 administered 48 hr after the initial immunization. As shown in Fig. 6 *A*, a single injection of CTLA-4Ig (100 μg) suppressed the first episode of clinical disease, but not the subsequent relapse. On the other hand, one injection of αCD80 (100 μg) 48 h after the primary immunization resulted in almost complete suppression of clinical disease. Conversely, one injection of αCD86 did not suppress disease, but rather resulted in modest disease exacerbation (Fig. 6 *B*). Finally, administration of both αCD80 plus αCD86 resulted in a delay in disease onset and reduced disease severity (Fig. 6 *C*), although disease suppression was not as complete as with αCD80 alone.

Discussion

In this study, we examined the role of the members of the B7 family, CD80 and CD86, in the prototypic, T cell-mediated, autoimmune disorder, EAE. There were several unexpected observations, the most notable being the unusual dose response of anti-CD80 plus anti-CD86 mAbs for inhibition of MBP-induced costimulation, and the observation that CTLA-4Ig itself can either exacerbate or inhibit disease, depending on the schedule of administration.

First, following in vivo priming of mice with MBP, we asked whether either CD80 (B7-1) or CD86 (B7-2) was predominantly responsible for providing the costimulation for MBP-specific LNC. Neither αCD80 or αCD86 alone significantly inhibited the in vitro proliferative response to MBP (Fig. 1 *A*). Although it has been suggested that CD86 may be the predominant costimulatory molecule in the B7 family (6, 51, 52), blocking of either CD80 or CD86 alone had little effect on the proliferative response in vitro. In addition, unlike the dose-dependent inhibition seen with CTLA-4Ig, the combination of αCD80 plus αCD86 resulted in the greatest inhibition of proliferation when present at the lowest dose tested. To test whether this observation might be due to signal transduction through the B7 molecules themselves, we repeated the experiment using Fab fragments (Fig. 1 *C*). Similar to CTLA-4Ig, using the Fab fragments, a dose-dependent inhibition of T cell proliferation was observed, suggesting that the antibodies may be signalling through the B7 receptors. Future experiments will attempt to further define the possibility of signalling through B7 molecules.

We next examined whether there was a difference in the ability of anti-B7 reagents to inhibit the proliferative response of MBP-specific LNC versus T cell lines. Prior studies regarding the activation requirements of naive and memory T cells in vitro have produced conflicting results. One study demonstrated that naive T cells required a second signal to produce IL-2, while previously primed T cells needed only peptide-MHC complex stimulation to produce IL-2 (46). Another study reported somewhat similar findings in that memory cells were less dependent than naive cells on accessory cell costimulation (47). In contrast, it has been suggested that dependence on costimulation is related more to the lymphokine secretion profile and less to the T cell's activation state (13). Our results are in agreement with the former observations. Following in vitro stimulation of MBP-primed LNC, restimulation of the MBP-specific T cells was not significantly inhibited (< 25%) by CTLA-4Ig or the combination of αCD80 plus αCD86 (Fig. 2, *A* and *B*). Despite the fact that these encephalitogenic, MBP-specific T cells are of the Th1 phenotype, it appeared that these cells became less dependent on B7 costimulation, presumably due to their prior stimulation in vitro and/or in vivo. Similar observations have been made on the requirement of encephalitogenic T cells for

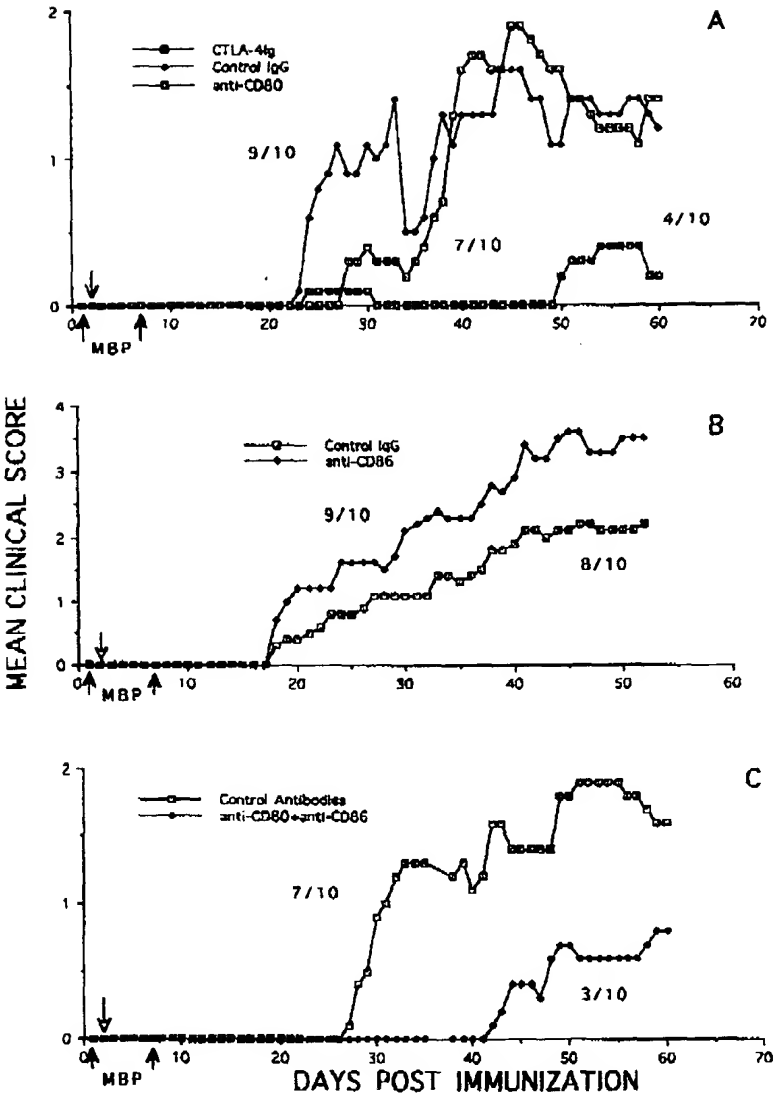

Figure 6. Administration of anti-B7 reagents in actively induced EAE. Groups of ten (PL × SJL)F1 mice were immunized with MBP/CFA on day 0 and re-immunized 7 d later (indicated by thick arrows labeled MBP). Two days after the initial immunization (indicated by *thin arrow*), mice received an i.p. injection of 100 μg of either CTLA-4Ig, anti-CD80 or mouse IgG2a *(A)*, anti-CD-86 or rat IgG2a *(B)*, or both anti-CD80 and anti-CD86 or control antibodies *(C)*. Mice were examined daily for clinical signs of disease and a mean clinical score assigned. In *A*, significant treatment differences were noted between control IgG and CTLA-4Ig ($P < 0.05$) and between control IgG and anti-CD80 ($P < 0.01$). Significant differences were also observed in *B* ($P < 0.05$) and in *C* ($P < 0.01$). The incidence of clinical disease during the observation period is indicated next to the corresponding clinical course.

costimulation through the CD4 molecule (53). Lymph node cells sensitized to MBP were much more sensitive to the inhibitory effects of anti-CD4 on their proliferative response than MBP-specific T cell lines, suggesting that T cell lines were less dependent on an interaction through the CD4 molecule. As we had previously demonstrated for CTLA-4Ig (7), the combination of αCD80 plus αCD86 during antigenic stimulation in vitro did not result in tolerance induction at the dose used, as upon subsequent antigenic stimulation with MBP these T cells produced IL-2 (Fig. 3 *C*).

The addition of anti-B7 reagents during the activation of MBP-specific LNC in vitro and in vivo did have differing effects on their encephalitogenicity (Figs. 4 and 6). The presence of αCD80 during in vitro activation had no effect on subsequent encephalitogenicity. αCD86 presence during in vitro activation of MBP-specific LNC did not inhibit the initial episode of clinical disease, but animals that received these cells did not develop the severe relapse suffered by animals that had received cells activated in the presence of control antibody. It is possible that a specific population of T cells or APC may be depleted following administration of anti-B7 antibodies. This observation could also be consistent with a model whereby unopposed CD80 costimulation results in a negative signal, and therefore, diminished disease severity. Alternatively, such cells may become insensitive to CD86 costimulation, relying on other pathways, so that subsequent CD86 costimulation does not reactivate these cells. This would be a particularly intriguing possibility in the EAE model, where the extracellular domain of CNS molecules such as myelin-associated glycoprotein and the axonal glycoprotein TAG-1 display homology with the extracellular domain of B7-2 (6). Thus, EAE-inducing cells localized to the CNS might utilize molecules homologous to B7-2. Such a possibility might also suggest that αCD86 might be effective in preventing disease relapses, if such costimulation played a role in reactivating encephalitogenic T cells.

Because addition of both αCD80 plus αCD86 at the doses used did not completely inhibit T cell encephalitogenicity (Fig. 4 *C*), it was possible that the inability to completely inhibit activation was due to costimulation provided by another costimulatory pathway. Alternatively, because of the in vivo priming, these cells were, in fact, no longer naive and required less costimulation. To examine whether in vivo administration of

CTLA-4Ig could inhibit the in vivo priming of naive lymphocytes, we gave CTLA-4Ig every other day for 10 injections, starting one day before the first immunization in an active model of EAE. Paradoxically, animals that received CTLA-4Ig actually developed more severe disease while consistent with our previous results, a single injection of CTLA-4Ig resulted in substantial clinical protection. Interestingly, transgenic mice that expressed a soluble form of murine CTLA-4 demonstrated enhanced expansion of antigen-specific CD4+ T cells (54). In that report, animals that received two immunizations separated by one week developed the most dramatic expansion of antigen-reactive cells, which is very similar to our model used. This suggested that frequent administration of CTLA-4Ig, or expression of such a transgene, resulted in the use of other costimulatory pathways. Alternatively, the constant presence of CTLA-4Ig may prevent the delivery of a subsequent negative signal via B7 ligands (36–38, 55).

To differentiate these possibilities, we performed a series of experiments where we gave only one injection of CTLA4-Ig or αCD80 and/or αCD86 48 h. after the initial immunization with MBP. This time point was chosen because this would be when B7-1 expression would be upregulated, but significantly after the peak expression of B7-2. In addition, this time was previously found to be optimal for the induction of allograft tolerance (56). Interestingly, one injection of CTLA-4Ig resulted in inhibition of the first episode of disease, yet subsequent relapses were unaffected (Fig. 6 *A*). αCD80, administered at the time this ligand would be expected to be upregulated, resulted in dramatic inhibition of clinical disease. In contrast, one dose of αCD86 mildly exacerbated clinical disease. This result is consistent with the recent observation that αB7-1 administered over several days reduces the incidence of EAE, while αB7-2 increased disease severity (57).

These results suggest several possibilities. One is that inhibition of CD80's initial costimulation blocked an important factor related to the development of encephalitogenicity. This could include the development of an MBP-specific T cell of the Th1 phenotype, which appears to be necessary to transfer EAE (8, 9). Such a possibility has recently been suggested for results in the EAE model (57), however in another model of autoimmune disease, the nonobese diabetic mouse model, αB7-2 treatment was protective while αB7-1 accelerated disease (58). This would suggest that the concept that B7-1 costimulation preferentially results in development of Th1 cells, while B7-2 results in the development of Th2 cells may be overly simplistic. Alternatively, allowing certain APC populations to give initial CD86 costimulation, which should be expressed as early as 6 hr. after the antigenic stimulus, may activate encephalitogenic precursors through CD28, but then subsequent inhibitory signals which might be delivered through CTLA-4 may have been blocked by the administration of αCD86 at 48 hr. after the initial immunization. Finally, our results suggest that the B7 receptors themselves may transduce signals, given the unusual dose response observed for the inhibition of MBP-induced T cell stimulation. Our results demonstrate that complex costimulatory interactions occur in immunopathologic states such as EAE.

Acknowledgments

This work is dedicated to the memory of Dale E. McFarlin.

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